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Investigating pheromone variability in a terrestrial woodland salamander

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INVESTIGATING PHEROMONE VARIABILITY IN A TERRESTRIAL WOODLAND SALAMANDER

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DEDICATION

To my parents, who never cease to amaze me.
Thank you for your love and support, as well as
the opportunities your hard work has afforded me.

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TABLE OF CONTENTS

TABLE OF CONTENTS (CONT'D)

LIST OF TABLES AND FIGURES

LIST OF TABLES AND FIGURES (CONT'D)

ABSTRACT

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The communication system of Plethodontid salamanders relies heavily upon chemical signaling. Pheromone molecules convey a tremendous amount of information to conspecifics, and facilitate a wide variety of the essential social functions of these animals. Much of the information contained within the pheromone mixture is variable from one individual to the next, such as the ability to recognize specific individuals, the size of the sender, and the relative "quality" of the signal. In order for variable information to be conveyed through chemical signals, the corresponding variability must be contained within the pheromone mixture itself. This variability may exist as a gradient of total volume, changes in the relative ratios of components, and even structural variability of the specific pheromone molecules. These aspects of pheromone variability will be explored in two Plethodontid salamanders (*Plethodon cinereus* and *P. shermani*) using biochemical methods and the phenomenon of female mate-choice as a behavioral measure.

I. INTRODUCTION

Plethodontid salamanders have been used extensively as a model system for studying pheromone communication, in part because they rely heavily upon chemical signals as opposed to other sensory modalities (Duellman and Trueb, 1994). Of these, the Eastern Red-backed salamander (*Plethodon cinereus*) has been the cornerstone of the majority of behavioral studies investigating the sociobiological roles played by pheromones (Jaeger, 1986). In Caudate amphibians, pheromone signaling is an essential form of communication that facilitates a tremendous assortment of both inter- and intraspecific functions.

Interspecifically, pheromones are used for: predator avoidance (Maerz et al., 2001; Sullivan et al., 2005; Murray and Jenkins, 1998), prey identification and location (Dickens, 1999; Placyk and Graves, 2002), heterospecific space allocation (Thurow, 1975; Quinn and Graves, 1999), and species recognition (Jaeger and Gergits, 1979; Dawley, 1984b; 1986b). Intraspecifically, pheromones aid in: establishing and maintaining territories (Jaeger, 1986), locating and aggregating with mates (Kikuyama et al., 1995; 2002; 2005), homing and orientation (Madison, 1969; Jaeger et al., 1986), aiding in mate assessment and mate-choice (Marco et al., 1998; Mathis, 1991), and even increasing the receptivity of the opposite sex (Houck, 1998; Houck et al., 1998; Rollman et al., 1999; Gergits and Jaeger, 1990b).

Decades of behavioral experiments have elaborated upon the vast degree of complexity with which salamanders communicate, but there are still large and looming questions that remain unanswered. The specific objectives for each experimental section of this research will be discussed in Section II, while the general focus of the research will be provided at the end of this Introduction. Prior to an understanding of the full scope of this Thesis, however, a great deal of background information will be necessary. The relevant biology of the model system (*Plethodon cinereus*) will hereby be provided.

I.A. BIOLOGICAL BACKGROUND OF THE EXPERIMENTAL SYSTEM

I.A.1. HABITAT, FORAGING AND ECOLOGY

Plethodon cinereus is a completely terrestrial lungless salamander that inhabits the deciduous forest floors of eastern North America. Adults range in size from 32 mm Snout-Vent Length (SVL) to a maximum size of roughly 52 mm SVL (Jaeger, 1986). Some researchers have reported no sexual size dimorphism (i.e. Sayler, 1966), while others have found statistically significant differences between sexes: some researchers claim that males obtain slightly larger sizes (see Nagel, 1977), while others indicate that females do (Petranka, 1998).

Plethodon cinereus are very abundant, and have even been claimed to be the most abundant vertebrate in its geographic region (Harding, 1997). Mathis (1991) reported population densities of 2.8 individuals/m^2 in Virginia. A study by Burton and Likens (1975) explored population densities in the Hubbard Brook Experimental Forest of New Hampshire. This study found a population density of

2950 salamanders/ha, a density of 0.295 salamanders/m^2. Interestingly, this indicates that the NH population is almost exactly an order of magnitude less densely-crowded than the VA population (which has been the most extensively studied in terms of behavior; see Jaeger, 1986). Also of note is that this assessment was conducted in 1975, and several studies have since documented decreasing population sizes throughout the extent of the biogeographic range (see Messere and Ducey, 1998). All population estimates are thought to be underestimated because only a small proportion of the population (2-32%) are above ground at any time (Taub, 1961). Even so, *P. cinereus* in NH was shown to compose the vast majority of the salamander biomass of the area (93.5%), which itself was greater than the biomass of all bird species, and about equivalent to that of mammals (Burton and Likens, 1975). This high density of animals leads to increased competition for spatial resources, and thus *P. cinereus* is highly territorial. Individuals of both sexes establish and defend territories under cover objects such as rocks and logs on the forest floor (Jaeger et al., 1982).*

Red-backed salamanders inhabit mainly deciduous forest, but can also be found in habitats mixed with conifers. Animals inhabit the moist leaf-litter when they are not present under forest floor cover objects or inside of decaying logs and stumps (DeGraaf and Yamasaki, 2001). *P. cinereus* prefer cool, loose and moist soil, with the pH originally thought to be a limiting factor. Recent research has since shown that healthy populations can tolerate surprisingly acidic soils (Moore and Wyman, 2010). Moisture, however, is a predominant limiting factor. As lungless

* For a full description of Territoriality, see Section I.A.3

salamanders (Plethodontids), all respiration occurs through the integument; thus, an adequate amount of moisture is required to enable sufficient respiration (Duellman and Trueb, 1994).

Plethodon cinereus is not tolerant to freezing, as some amphibians are. Behavioral mechanisms are therefore employed to avoid freezing during the winter (Storey and Storey, 1986), such as residing at depths up to a meter into the soil, in the interior of stumps, logs or ant mounds (Petranka, 1998). The extent of winter inactivity is unclear; there are several accounts of activity in the winter months, particularly in mild weather (DeGraaf and Yamasaki, 2001). The general trend seems to be hibernation occurring in the late fall and lasting until snowmelt. *P. cinereus* is known to be able to withstand long periods without food, which enables hibernation when coupled with reduced body temperature (Feder, 1983).

Predators of the red-backed salamander are mainly snakes and small mammals, though occasionally birds. The Spotted salamander (*Ambystoma maculatum*) is also known to occasionally prey upon red-backs (Ducey et al., 1994). Eastern Garter snakes (*Thamnophis sirtalis*) and the Short-tailed shrew (*Blarina brevicauda*) are among the main predators. Plethodontids are armed with an assortment of anti-predator mechanisms. The preliminary line of defense is the use of chemical cues left by predators, to simply avoid contact (Murray and Jenkins, 1999; Sullivan et al., 2005). Additionally, the entire integument is known to produce a variety of noxious chemicals (see Duellman and Trueb, 1994). These products make *P. cinereus* salamanders unpalatable to a variety of potential predators. The lateral and dorsal regions of the tail also secrete highly adhesive molecules that

make physical acquisition by the predator very difficult (Brodie et al., 1979; Petranka, 1998). These chemicals are said to be able to temporarily glue the mouth of a snake shut, and are thought to be composed of a mixture of mucopolysaccharide and proteinaceous components (Largen and Woodley, 2008). Lastly, like some lizards and other salamanders, Plethodontids are capable of tail autotomy, the ability of the tail to detach itself quickly from the rest of the body (Wake and Dresner, 1967; Wise and Jaeger, 1998; Wise et al., 2004). The bulk of the energy reserves of Plethodontids are located in the tail (Jaeger, 1981; 1986), and these reserves enable the tail to continue moving and releasing the adhesive secretions of the caudal glands, increasing the chance of a successful escape.

Prey items of *P. cinereus* consist of a wide assortment of invertebrates, including (but not limited to): small insects and larvae, worms, mollusks, spiders, mites, millipedes. There are even reports of occasional cannibalism of eggs and juveniles (DeGraaf and Yamasaki, 2001; Burton, 1976). Burton (1976) analyzed the gut contents of the New Hampshire population (Hubbard Brook) and found that the total insect weight (larval and adult) consisted of 85.3% of the entire diet. Of this 85.3%, most of the insect prey consumed was in larval form. Of non-insect prey items, preference seems to be placed upon soft-bodied animals (Walls et al., 1989; Jaeger, 1990; Gabor and Jaeger, 1995; Maerz et al., 2005). Soft-bodied animals pass more quickly through the gut than harder, more chitinous prey items. Thus, digestion efficiency is higher, in addition to happening more quickly which allows the animals to increase the overall rate of energy intake (Walls et al., 1989; Gabor and Jaeger, 1995; Bobka et al., 1981; Jaeger and Barnard, 1981).

Rainfall is a limiting factor for the ability for *P. cinereus* to forage outside of its territory (Jaeger, 1978; Jaeger, 1986). Red-backed salamanders are also not well-adapted for sub-surface foraging (Fraser, 1976), and therefore rainfall provides an opportunity to increase foraging search times to locate more desirable prey (Jaeger, 1978). As rainfall lessens, the leaf-litter begins to dry, while cover objects conserve moisture. Cover objects also provide a reliable food patch for prolonged dry periods, and this contributes to these objects being used by both sexes as territories; the territory owner ensures sole use of the food patch by excluding competitors (Jaeger et al., 1982). Even so, Jaeger (1972) found that during long dry periods, most salamanders have few or no prey items in their gut. This situation led to what he described as frequent bottleneck periods of prey availability.

Jaeger (1980a) identified distinct differences in foraging abilities between individuals (positive vs. negative energy-budgets) during both wet and dry periods. Since certain patches differ in the quality of prey, and the availability of prey can become drastically reduced for prolonged periods (lack of rainfall), there is intense competition for access to quality food patches (Jaeger, 1986).

I.A.2. MATING SYSTEM AND REPRODUCTION

Mature males attempt courtship annually, while females differ in their mating patterns between populations: females mate biennially in the North*, but have been known to court annually in the southern reaches of its range (Sayler, 1966; Petranka, 1998). Throughout the geographic range, the reproductive season is from September to November in the fall, and again from April to June in the spring

* Though this is not true in all cases (see Lotter, 1978).

(Duellman and Trueb, 1994). However, there is still some ambiguity regarding the boundaries of the mating season, as some consider it to be a continuous, prolonged courtship season from the fall through the spring (Sayler, 1966; Dawley et al., 2000). Females store sperm from previous matings in the fall and spring in the spermatheca (Sever, 1978a). Eggs are fertilized internally at the moment of oviposition, which occurrs in late spring (May-June; Sayler, 1966). Clutch sizes range anywhere from 1-15 eggs, with a typical clutch size between 6-9 eggs (Lotter, 1978; Petranka, 1998).

Female fecundity is positively correlated with body size (SVL) in *P. cinereus*: larger females carry larger clutches on average (Nagel, 1977; Lotter, 1978; Fraser, 1980). This trend has also been shown in other Plethodontids (Verrell, 1995; Peacock and Nussbaum, 1973; Duellman and Trueb, 1994). Similarly, male testicular lobes have been shown to accrue with age, and thus older (and larger) males have a higher reproductive potential. It is therefore in the best interest of each sex to select larger mates in order to maximize fitness (Duellman and Trueb, 1994).

After oviposition, females provide parental care in the form of guarding the eggs within her territory for 6-8 weeks while the eggs develop (Petranka, 1998). Males can also occasionally be found cohabitating with an egg-guarding female (Friet, 1995), and thus some form of paternal care can also be present. Eggs undergo direct development; the young emerge morphologically identical to the adult form (Duellman and Trueb, 1994). The young are thought to stay within the nest for 1-3 weeks after hatching, which usually takes place in August-September (Petranka, 1998). Juveniles reach sexual maturity after two years of growth (Sayler, 1966) at a

size range of 32-37 mm SVL (for males) and 34-39 mm SVL (for females). Females typically will not reproduce until the third year (Lotter, 1978).

Jaeger (1986) has suggested a maximum life span of about 18 years, and has identified the life-history similarities between *P. cinereus* and a typical "k-selected species," including: an extended age to maturity, iteroparity with small clutch sizes, parental care, and a relatively long life-span.

The mating system is thought to be based on female choice (Duellman and Trueb, 1994), though both males and females will establish clear preferences for a variety of characteristics. Red-backs are considered socially monogamous (Gillette et al., 2000a), though this is not necessarily true for all individuals. Even in cases where social monogamy is evident, it is not always indicative of true genetic monogamy (Liebgold et al., 2006). A large degree of "sexual coercion" takes place: males have been shown to "punish" socially polyandrous females (Jaeger et al., 2002), while females have also been shown to "punish" socially polygynous males (Prosen et al., 2004). This coercion is manifested through agonistic display behaviors and also appears to be based upon information acquired via pheromone signals (Prosen et al., 2004; Jaeger et al., 2002; Gillette, 1998).

The entire mating process is largely facilitated through the use of both territorial and courtship pheromones. The preliminary stages of courtship are based upon mate assessment, in which both sexes identify the characteristics of the opposite sex through visual inspection, but also through volatile chemical signals, and further inspection on non-volatile chemical signals deposited on the substrate (Dantzer and Jaeger, 2007a; 2007b). These pheromones provide a vast array of

information to conspecifics (described in detail in Section I.A.4), all of which is used to facilitate mate assessment (Mathis, 1990a; Marco et al. 1998; Gillette et al., 2000b).

Courtship in Plethodontids has been well-documented, and the particular behaviors seem to be well-conserved across taxa (Arnold, 1977).* The general sequence of Plethodontid courtship is as follows. First, the male (typically) becomes aware of a potential mate, approaches and begins frequent nudging behavior and rubbing of the female with his snout (the location of the chemosensory receptors). The female may also engage in frequent snout contact with particular regions of the male's body. This portion of the courtship ritual can last for varying lengths of time (both between and within species), though in *P. cinereus*, it lasted for an average of 22 minutes (Dyal, 2006). Depending upon the receptivity of the female, the male will then commence the "tail-straddling walk." This courtship ritual consists of the male leading and walking forward, while the female follows closely behind making prolonged contact with the dorsal base of the male's tail with her snout (Petranka, 1998; Duellman and Trueb, 1994). The contact of the female's snout with the Dorsal Tail Base of the male presumably allows for the transfer of integumentary pheromones to the female (Simons and Felgenhauer, 1992; Palmer et al., 2007a). The Dorsal Tail Base is one of two main courtship glands, the other of which is the Mental Gland.**

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* Dyal (2006) provides an excellent comparison of *P. cinereus* courtship behaviors to that of two other species (*P. angusticlavis* and *P. richmondi*).

** For a full description of the glandular regions responsible for pheromone production (and their corresponding functions) see Section I.A.6.

At this point, the male will administer courtship pheromones to the female's nares from his Mental Gland in one of two ways, depending upon the species in question. Some Plethodontids (such as *P. jordani/shermani*) will perform a "chin slap" behavior, in which the male turns backward (while the female is still in the tail-straddling position) and quickly presses the underside of his chin (the Mental Gland) to the female's snout and thus her chemosensory receptors. This mode of pheromone delivery has been coined "slapping" or "olfactory" delivery.

Other species, including *P. cinereus*, perform a "scratching" mode of pheromone delivery (also called the "vaccination" mode, e.g. Watts et al., 2004; Palmer et al. 2005). In this case, the male uses his protruding pre-maxillary teeth to scratch the skin of the female, and then rubs the area of his Mental Gland over the recently opened surface, directly administering the courtship pheromones to the female's bloodstream (Duellman and Trueb, 1994). This is considered the ancestral Plethodontid mode of pheromone delivery, while the "slapping" mode of delivery is thought to have been derived ~42 MYA (Palmer et al., 2007b).

After Mental Gland pheromone delivery, the male continues to walk forward, undulates his tail from side to side, and eventually deposits a spermatophore from his cloaca. If completely receptive, the female will continue forward and uptake the spermatophore with her cloaca. In *P. cinereus*, this stage lasted for an average of ~6 minutes (excluding males who deposited multiple spermatophores, which can occasionally happen). The longest stage of the entire courtship is the initial "persuasion" stage (Dyal, 2006).

As demonstrated above, pheromone communication plays an integral role in facilitating mating - from the assessment of potential mates to the completion of the courtship ritual*. The particular focus of this research will be placed upon the former, though an understanding of the latter stages of courtship is also highly pertinent. Information contained within the pheromone mixture allows for the honest assessment of the characteristics of a conspecific, but this information is present in the public domain, and is not utilized only for the purposes of mating. Territorial advertisements can also be used for intrasexual competitor assessment in agonistic encounters, and it is to this area of research that we now turn.

I.A.3. HOME RANGE, TERRITORIALITY AND AGGRESSION

Eastern red-backed salamanders of both sexes establish territories that are aggressively defended, both in and out of the breeding season (Jaeger et al., 1982; 1986; Jaeger, 1986; Mathis, 1989; Gabor and Jaeger, 1995). Territories of *P. cinereus* have been identified by Mathis (1991) as 0.163 m^2 areas. These territories are aggressively defended (see below), and both conspecific and heterospecific individuals are excluded (Jaeger, 1971; 1972; 1974). Home range sizes of *P. cinereus* have been calculated as: 12.97 m^2 for males, 24.34 m^s for females, and 12.87 m^s for juveniles (Kleeberger and Werner, 1982).

Red-backed salamanders exhibit an impressive degree of site-attachment to their territory (Gergits and Jaeger, 1990a; Kleeberger and Werner, 1982). Gergits and Jaeger (1990a) also reported that 90% of recaptures took placed within one meter of the original capture. Incredibly, Gillette (2003) found that over a three year

* See Section I.A.4 for information on the role of pheromones in mate-assessment.

period, salamanders (n=3487) hardly ever moved from one territory (30 x 30 cm) to another (only 4 m away).

Not only are their territories firmly established at small scales, red-backs also return to their territories when experimentally displaced, even over large distances (*P. cinereus* - Kleeberger and Werner, 1983; *P. jordani* - Madison, 1969; Madison and Shoop, 1970). Kleeberger and Werner (1982) found that return rates for animals displaced 15 m were 100%, while animals displaced 30 m were 80% successful, and animals displaced 90 m were 25% successful in returning. They described the route of return as direct, and usually taking place within 24 hours of displacement. This relatively impressive homing behavior is thought to be facilitated through olfactory cues, mainly by recognizing the scent of familiar individuals (Madison, 1969; Jaeger, 1986; see Section I.A.4).

Territoriality in *P. cinereus* stems largely from intense competition that arises from two main factors: high population densities (Mathis, 1991; Burton and Likens, 1975; Jaeger, 1986), and fluctuating prey availability (Jaeger, 1972; 1978; 1980a; 1986; 1990; see Section I.A.1). In addition to (or likely because of) this competition for defense of reliable food patches, the maintenance of a territory also contributes greatly to accessing mates (Walls et al., 1989; Jaeger and Wise, 1991; Mathis, 1990a; see Section I.A.1). Because of this, not all territories are occupied by a single salamander, but that frequently the territory will be defended by a male-female pair (Jaeger et al., 1995; Lang and Jaeger, 2000). Jaeger et al. (1995) reported that this type of cohabitation accounted for 28% of all salamander encounters. Knowing that red-backed salamanders frequently engage in social monogamy (Gillette et al.,

2000a; Jaeger et al., 2002; Prosen et al., 2004), this result is not surprising. Conversely, while not all territories are maintained by a single individual, some individuals do not have possession of a territory at all. These individuals ("floaters"; Gabor and Jaeger, 1995) are forced to remain in the leaf-litter or go underground in sub-surface tunnels (Taub, 1961; Jaeger, 1986). Sub-surface foraging is poor compared to foraging in the leaf-litter (Fraser, 1976), and these "floaters" have been shown to maintain lower energy budgets than territory-holders (Gabor and Jaeger, 1995; Jaeger et al., 1981; Mathis, 1991).

Competition for territories is facilitated through territorial scent-marks (see details in Section I.A.4), but can eventually become directly manifested through the use of distinct agonistic behaviors (Jaeger, 1984; Jaeger and Schwarz, 1991; Jaeger, 1981; Horne, 1988; Thurow, 1975; Gergits and Jaeger, 1990b; Jaeger et al., 2005). Jaeger (1984) and Jaeger and Schwarz (1991) provide particularly good descriptions of agonistic interactions between red-backed salamanders, and these behaviors will be summarized here in brief.

One subset of agonistic behaviors of the red-backed salamander consists of aggressive and submissive postures. The aggressive threat postures have been coined "Front of Trunk Raised" (FTR) and "All Trunk Raised" (ATR) by Jaeger (1984). Jaeger and Schwarz (1991) refined this initial description into five categories of ATR behavior (ATR 1-5), and explained the progression as a graded signal indicating the level of threat. The initial level of threat (originally termed FTR) begins with lifting the front of the animal's trunk off of the substrate, where it remains for resting postures. The next levels of the graded signal constitute the

lifting of the remainder of the trunk, and eventually the tail off the ground. The highest level of threat consists of the entire trunk and tail raised off of the ground, with both the back and tail arched substantially upwards. Jaeger and Schwarz (1991) described this as the "typical vertebrate 'look big' threat posture." The corresponding submissive posture has been called "Flat," in which the entire length of the body (head, trunk and tail) are pressed flat against the substrate. Jaeger (1984) also considered the orientation of the gaze ("Look Toward" and "Look Away") as aggressive and submissive posture (respectively).

The behavior of "Nose Tapping" (NT) should also be mentioned (though it will be addressed in more detail in Section I.A.5). This behavior consists of an arching of the neck, which orients the snout towards the ground, at which point it is pressed to the substrate. This behavior is a means of obtaining non-volatile chemical cues from the substrate, and is frequently observed in both agonistic and courtship encounters (Jaeger, 1981; 1984; Dantzer and Jaeger, 2007b; Schubert et al., 2008). A related set of behaviors were coined "Chin Tapping" and "Cloacal Tap" by Jaeger (1984). These behaviors consist of the corresponding region of the body being pressed deliberately against the substrate. Each of these glandular regions play an essential role in chemical signaling, and these behaviors can be observed during agonistic bouts. The latter behavior was coined the "Postcloacal Press," and has since been shown to be the site of production of territorial scent-marks (Simon and Madison, 1984; Simons and Felgenhauer, 1992; Simons et al., 1994; 1999)*. The postcloacal region of the body is often assessed by an intruding salamander,

* See Section I.A.6 for information pertaining to pheromone production.

apparently at the permission of the resident, prior to the engagement of any actual threat posturing (Jaeger and Gabor, 1993; Simons et al., 1997). This probably allows for scent-matching (Gosling, 1990) by the intruder to ensure the identity of the resident is correct (Simons et al., 1997; and Jaeger, 2007a).

Lastly, the greatest degree of red-back aggression is through the use of biting (Jaeger, 1981; 1984). Jaeger (1981) observed that bites were almost always directed towards the tail (the fat storage organ) or the snout (the chemosensory receptors). Between these two regions, the snout accounted for twice as many bites as the tail. The snout is the location of the chemosensory receptors, which are used to locate predators/prey, and to detect the pheromones of conspecifics. Directed attacks often resulted in damage and scarring of this region, which can seriously hinder an individuals chances of successfully completing these vital activities. The tail region is also the location of the vast majority of fat storage in salamanders (Duellman and Trueb, 1994). Also, since tail autotomy is possible, these attacks can cause the loss of a large supply of energy. Indeed, field observations have implied that intraspecific aggression can lead to tail autotomy (Jaeger, 1981). Tail condition is known to act as a status signal: tail autotomy has been shown to drastically reduce the Resource-Holding Potential (RHP; Maynard-Smith and Parker, 1976) of a territory holder, and it is thought to affect mating opportunities (Wise and Jaeger, 1998; Wise et al., 2004). In short, bites are specifically directed at regions of the body that can seriously hinder the fitness of the attacked salamander.

Intersexual aggression has been shown to be less intense than intrasexual aggression (Lang and Jaeger, 2000); *Plethodon* cinereus are more aggressive to

members of the same sex (Jaeger, 1984; Horne, 1988). This is most likely because same-sex individuals are competitors for all types of resources (including reproductive resources), while opposite-sex individuals are in and of themselves a reproductive resource.

The size of the salamander and its level of aggression are key factors in determining the outcome of an agonistic encounter (Mathis, 1990b). Large salamanders are known to be more aggressive, and thus have access to higher-quality territories and mating opportunities (Mathis, 1991). Residents are also more aggressive than intruders and have a much higher chance of maintaining the territory (*P. cinereus* – Jaeger et al., 1982; Nunes and Jaeger, 1989; Simons et al., 1997; *P. angusticlavus* - Mathis et al., 2000). Jaeger et al. (1982) found that intruders left the territory (before the resident even escalated the contest to biting) in 83% of cases. Additionally, aggressive behavior increases with the length of time that a resident holds a territory (Nunes and Jaeger, 1989). Eastern red-backed salamanders also exhibit "Dear Enemy Aggression" Jaeger (1981), with levels of aggression being lessened towards familiar individuals (i.e. a neighbor).

The quality of the territory itself is an important factor in modulating the level of aggression in agonistic bouts between male red-backed salamanders. Gabor and Jaeger (1995) tested this idea by manipulating the quality of the diet fed to males prior to agonistic encounters. The highest levels of aggression took place with males that were fed softer-bodied prey items (i.e. termites), as opposed to those that were fed hard-bodied prey with large proportions of chitin (i.e. ants).* Older (and

* See Section I.A.1

thus larger) males are more likely to possess higher-quality territories (Mathis, 1990b; 1991; Gabor and Jaeger, 1995; Houck, 1988). The causality of this situation remains in question; it is unclear whether high-quality territories are simply obtained by larger males, or if the males are larger because they possess high-quality territories. It is most likely a positive feedback situation, in which both cases are true; larger males are more likely to obtain high-quality territories, but these territories then contribute to them becoming even larger and more competitive, thereby further increasing their already high Resource-Holding-Potential.

Jaeger (1986) outlined (via Gergits, 1982) the requirements of a properly-defined system of salamander territoriality. Territorial salamanders must have "a fixed site (or sites) that is advertised (as by pheromones) and defended, and from which there is a high probability of expelling an intruder." It is clear that all of these requirements are met in the case of *Plethodon cinereus,* but to this point the use of pheromones for territorial advertisement has been largely unaddressed. This area of research will now be addressed in full in the following section.

I.A.4. PHEROMONES AS TERRITORIAL ADVERTISEMENTS

Pheromones are used by territorial Plethodontid salamanders to convey a vast array of information. The chief function of territorial advertisements is to provide information about the territory owner (Simons et al., 1997). Pheromones have been shown to include information about the species (Jaeger and Gergits, 1979; Dawley, 1984b; 1986), sex (Jaeger et al., 1986; Dantzer and Jaeger, 2007a; Dawley, 1984a; 1984b), size/relative age (Mathis, 1990a; 1991; Marco et al., 1998; Verrell,

1995) and individual identity of the sender (Madison, 1975; Tristram, 1977; McGavin, 1978; Jaeger, 1981; Simon and Madison, 1984; Jaeger et al., 1986).

Further information can even be inferred about the morphological and physiological condition of the sender, such as: tail autotomy (Simons et al., 1997), injury (Sullivan et al., 2003), the reproductive condition of females (gravid vs. non-gravid; Dantzer and Jaeger, 2007b; Marco et al., 1998), the quality of male diet (Walls et al., 1989; Jaeger and Wise, 1991), and male parasite-load (Maksimowich and Mathis, 2001).

These associative and investigatory preferences were measured through blind behavioral experiments, in which there were no visual cues present from the conspecific salamander (the sender). Thus it becomes clear that the information on which the behavioral preference was based must be contained within the pheromone mixture itself. This type of analysis has elucidated what information receivers can and cannot infer *through the chemical medium* about the sender of the signal. Jaeger (1986) has outlined a synopsis of the general experimental designs used for testing this phenomenon in previous behavioral analyses.

Regardless of the sex of the receiver, it would always be in its best interest to be able to determine the sex of the sender; this would serve to minimize unwanted risk associated with intrasexual encounters, as well as to maximize intersexual encounters during the mating season. Indeed, both males and females of *P. cinereus* are able to determine the sex of the sender solely through chemical signals (Jaeger and Gergits, 1979; Dawley, 1984b; Jaeger et al., 1986; Dantzer and Jaeger, 2007a).

Prior to the more recent revelations regarding the complexity of pheromone signaling, territorial advertisements were initially thought to be used solely for marking a territory as owned, and to function in actively keeping out intruders. It was later found that pheromonal markers only actually deter a small proportion of intruders (Jaeger et al., 1982; Simons et al., 1997). Even though scent-marks are not a complete defense against territorial intrusions, they do play a large role in ensuring the successful defense of the resident. When residents were experimentally prohibited from scent-marking, residents were significantly less aggressive (Simons et al., 1994).

After these initial results, it was still unclear as to whether or not complex information about the sender was actually being conveyed through the pheromone medium, or if a more parsimonious explanation could be responsible. This simpler explanation was a "scent-matching" hypothesis (similar to the situation known with mammals; Gosling, 1990). In this hypothesis, scent-marks only provided the ability to match the identity of the scent-mark to that of the resident. If the scent-mark did not match the conspecific salamander, and intruder would be more likely to compete aggressively, because the salamander in question was not actually the resident. Simons et al. (1997) set out to compare this hypothesis to those that implied more complex pheromone communication. The conclusion of this analysis was that scent-marking is primarily used for providing information to conspecifics (although defense of territories against heterospecifics is also a crucial function), to be used in intrasexual competition. In a way this conclusion is far from surprising, based on the extensive research that had previously elucidated the ability of

pheromones to convey information about the species (Jaeger and Gergits, 1979; Dawley, 1984b; 1986b), size (Mathis, 1990a; 1991), individual identity (Madison, 1975; Tristram, 1977; McGavin, 1978; Jaeger et al., 1981; 1983; Jaeger, 1981), and even diet-quality of the sender (Walls et al., 1989; Jaeger and Wise, 1991).

The placement of territorial pheromone markers, however, does not only allow for the assessment of individuals for use in intrasexual competition. Pheromone markers also play a tremendous role in mate assessment, mate-choice and eventual courtship (see Section I.A.2). Prior to even beginning the courtship ritual, a putative reproducer is faced with the challenge of finding, informing and persuading a mate to commence. Again, these challenges are overcome through the use of chemical signaling. Specifically, females are strongly selected for a mate-choice preference for large males. Various Plethodontid females have been shown to prefer to investigate and associate with the scent of large males over that of small males (*P. cinereus* - Mathis, 1991; *P. vehiculum, P. dunni* - Marco et al., 1998; *Desmognathus santeetlah* - Verrell, 1995). Females of *Plethodon angusticlavus* have also been shown to prefer the scent of males with low-parasite loads (Maksimowich and Mathis, 2001). Both of these characteristics strongly affect the potential fitness of the reproductive female (see Section I.A.2), and thus the proximate mechanisms of this discrimination has been able to evolve.

Females are not only able to infer direct information about the morphological and physiological quality of the individual; they are also able to infer different types of *indirect* information about the environment in which the sender lives, and even information about the behavior of the male as well. For example, female *P. cinereus*

will establish a significant preference for the scent of males that have been raised on a high-quality diet over those that have been raised on a low-quality diet (Walls et al., 1989; Jaeger and Wise, 1991). This provides direct information about the physiological quality of the male: having a larger store of energy available to invest into mating and further resource acquisition. However, this can also provide indirect information about the foraging behavior of the individual, and even the competitive ability required to obtain those higher-quality resources over his peers, through agonistic encounters (i.e. Resource-Holding Potential). Additionally, not only does this provide insight into the proximate expressions of these abilities, it may also provide insight into the quality of the genetic predispositions that were (and are to be) inherited (i.e. a "good-genes" argument; Zahavi, 1975).

Males are also acted on by selection through the optimization of reproductive success, and should therefore have also developed proximate preferences for female traits that affect fitness. Male Plethodontids have thus also been shown to prefer the scent of larger females versus smaller females (Marco et al., 1998). There are positive relationships between female size and fecundity (Duellman and Trueb, 1994; see Section I.A.2), and thus larger females provide a higher potential fitness. Males have also been shown to prefer to associate with the scent of gravid (egg-bearing/reproductive) females versus non-gravid females (Dantzer and Jaeger, 2007b; Marco et al., 1998).

An important point to keep in mind (when considering intrasexual competitor and potential mate assessment) is not that these individuals can and do make these behavioral decisions (which align with the predictions of evolutionary

theory), but that all of the cited behavioral interactions have been made *through the detection of pheromone sources only*. This information must therefore be encoded within the pheromone mixtures themselves. At this point, we now turn to the mechanisms for perceiving pheromones, the glandular regions of production (and their various functions) and lastly, the biochemistry of the known Plethodontid pheromones.

I.A.5. PHEROMONE RECEPTION MECHANISMS

There are two adjacent systems for detecting olfactory cues in amphibians (Duellman and Trueb, 1994; Dawley and Bass 1988; 1989). The first is the main olfactory system, which utilizes the main olfactory chamber (MOC; also called the cavum principale). This system is used to detect mainly volatile olfactory cues (Dawley, 1992). The second system is the accessory olfactory system, which utilizes the vomeronasal organ (VNO; also called Jacobson's organ). The vomeronasal organ is a lateral diverticulae of the main olfactory chamber, and is used for the detection of non-volatile pheromone molecules (Dawley and Bass, 1989).

Plethodontid salamanders possess a unique adaptation that aids in the efficiency of detecting chemical cues in the substrate: the nasolabial grooves. These features are dorsolateral grooves that run along the rostrum and extend into the external nares. Inside the snout, the grooves enter the nasal cavity and terminate at the anterior region of the VNO (Dawley and Bass, 1989). These grooves act as capillary tubes when pressed to the substrate during the characteristic Nose Tapping behavior (Brown, 1968). Once the nasolabial grooves are pressed to the

ground, the non-volatile chemical cues present in the substrate travel along their path and are delivered directly to the VNO (Dawley and Bass, 1989).

Plethodontids also perform behaviors to increase the rate of volatile molecules into the MOC of the nasal organ. Muscles of the buccopharyngeal cavity are utilized to produce rapid oscillations which increase the rate of airflow to the MOC (Duellman and Trueb, 1994; Simons et al., 1997; Thurow, 1968). This behavior has been coined "gular pumping" (Simons et al., 1997).

Receptors on the surface of the two olfactory epithelia bind to their appropriate molecules, but these signals are projected along different (but parallel) routes to the central nervous system (CNS), depending on which olfactory system was activated. The MOC projects to the CNS via the olfactory nerve (cranial nerve 1) to the main olfactory bulb in the telencephalon, while the VNO projects to the accessory olfactory bulb of the telencephalon (Duelman and Trueb, 1994; Schmidt and Roth, 1990). Neurons of the accessory olfactory bulb then project to various regions of the brain involved in reproduction and physiology, such as: the medial amygdala, bed nucleus of the stria terminalis, the preoptic area and the ventromedial hypothalamus (Schmidt and Roth, 1990; Schubert et al., 2006).*

Various methods have been used to analyze the neuronal response to differing pheromone cues. The electro-olfactogram method (EOG; Toyoda and Kikuyama, 2000; Scott and Scott-Johnson, 2002) is a means of detecting the magnitude of the signal traveling along the cranial nerve, and thus the degree of response of the sensory organ itself. Other researchers (Trotier and MacLeod, 1982)

* For more information on the neurobiology of pheromone reception, see Laberge et al. (2008) and Dawley (1998).

have measured the intracellular responses of olfactory neurons. A more recent method has been employed to enable increased precision in assessing the response of the entire nasal organ. This method, the agmatine-uptake method, allows for counting the exact number of neurons that respond (Wirsig-Wiechmann et al., 2002; 2006). This method employs a modified amino acid that passes through generic cation channels during neuronal activation. Through the use of antibody labeling, it is possible to determine which neurons fired in the presence of certain chemical stimuli. These methods have provided a great deal of insight into the physiology of pheromone reception, as well as the functions of the pheromones themselves.

I.A.6. SITES OF PHEROMONE PRODUCTION

The first attempt (Jaeger, 1986) to identify the location of territorial scent-marks was conducted by Simon and Madison (1984). This study used both fecal pellets and cloacal washes to determine if an individual red-backed salamander could differentiate between the scent of itself and that of a conspecific: salamanders were able to make this distinction in both conditions. This not only verified the ability of *P. cinereus* to distinguish between the identities of pheromone signals, but narrowed the focus to the source regions from which this discrimination is made: a significant preference was found through the use of cloacal washes, indicating that the fecal pellet themselves were not necessarily the source of scent-marks. Indeed, the fecal pellet treatment would have contained both cloacal signals and fecal signals, while the cloacal wash treatment eliminated fecal signals. Thus, this was the first indication that the cloacal region was involved in producing scent-marks. This finding was later to be verified conclusively by Simons et al. (1994; 1999).

Jaeger et al. (1986) then followed up with this study to determine if fecal pellets outside of burrows were sufficient for discriminating between the scents of self, conspecifics and blank controls; this was shown to be possible. Several studies have since utilized fecal pellets in behavioral trials to test the ability of salamanders to infer certain types of information (e.g. diet-quality - Walls et al., 1989; size - Mathis, 1991; species recognition - Ovaska and Davis, 1992; parasite load - Maksimowich and Mathis, 2001). Even in cases where whole fecal pellets weren't exclusively used, substrates on which the salamander had the ability to mark for a prolonged period of time were utilized, and thus fecal pellets were likely present.

Regardless, several lines of evidence have now elaborated upon the role of the postcloacal region in the production of scent-marks. Sever (1978b) had previously suggested that granular glands on the ventral surface of the tail were likely to be pressed over the fecal pellets after release (Jaeger, 1986). Fecal pellets are also known to be coated with mucous prior to release (Duellman and Trueb, 1994). Additionally, cloacal vent glands (within the cloaca itself) are not present in females (Sever, 1978a), but several studies have since shown the ability of females to convey a comparable amount of information through territorial scent-marks (Mathis, 1990a; Dantzer and Jaeger, 2007b), including through fecal pellets (Mathis, 1990a). Jaeger (1981; 1984) also described the "Cloacal Tapping" behavior, in which salamanders of both sexes will deliberately press the ventral portion of the tail (just posterior to the cloaca) to the substrate while walking. This behavior has since been coined the "Postcloacal Press" and has since been shown to indeed be the source of territorial advertisement (Simons and Felgenhauer, 1992; Simons et al., 1994; 1999).

Two other glandular regions of the body also deserve mention: the Mental Gland (Sever, 1976b; Simons and Felgenhauer, 1992; Houck and Reagan, 1990; 1998) and the Dorsal Tail Base (Simons and Felgenhauer, 1992). Both of these glandular regions are thought to be mostly utilized in courtship (see Section I.A.2), though Jaeger (1984) did identify a relatively frequent behavior of "Chin Tapping," in which the region of the Mental Gland is pressed deliberately to the substrate.

Thus, different glandular regions of the body appear to function for different purposes in the behavioral physiology and sociobiology of Plethodontid salamanders. Few pheromone molecules have been explicitly identified (see Section I.A.8), but the cellular characteristics and structures of the integumental glands that produce these molecules have been elucidated (see Section I.A.7).

Lastly, new research has shed light on the different functions of volatile vs. non-volatile pheromones. Again, most of the original research used either whole fecal pellets, or allowed a salamander to mark a substrate for a prolonged time. Neither of these methods allows for the separation of volatile and non-volatile components; thus, volatile molecules themselves may be sufficient for some of the functions attributed to scent-marks as a whole. Martin et al. (2005) originally showed that residents could detect the scents of intruders solely through volatile cues. It was initially claimed that determination of sex was not possible through this medium, but a recent study (including some of the same authors, i.e. R.G. Jaeger) has shown that red-backed salamanders can infer the sex of the sender through volatile pheromone cues (Dantzer and Jaeger, 2007a). Dawley (1984b) has also demonstrated this with *P. jordani* and *P. glutinosis*. Dantzer and Jaeger (2007b) have

also shown that males can even infer the reproductive condition (gravid vs. non-gravid) of females through only volatile pheromone cues.

Chemical signaling theory holds that larger molecules offer an increase in structural variability, which can therefore encode a greater degree of information (Alberts, 1992). Thus, it may be that certain types of two-fold information (i.e. male/female; gravid/non-gravid) could be conveyed through small (volatile) molecules, but that more complex information (i.e. the identity of the sender) may require larger (and thus non-volatile) molecules. However, it has been implied that olfactory cues of individual recognition may be of great importance in the homing and orientation behavior of Plethodontids (Madison, 1969; Dawley, 1984b; Jaeger, 1986), though the role of volatile (vs. non-volatile) molecules is unclear.

I.A.7. GLAND HISTOLOGY AND COMPOSITION OF SECRETIONS

The integument of amphibians consists of an outer layer (the epidermis), and a lower layer (the dermis). The epidermis contains two distinct layers: the outer stratum corneum (one cell layer) and the inner stratum germinativum (4-8 cells thick). The dermis also consists of two layers: the stratum spongiosum (looser tissue with glands present) and the stratum compactum (densely-packed collagenous connective tissues). Within these integumental layers, there are three classes of secretory glands present: granular (or serous) glands, mucous glands, and lipid glands. All gland types are alveolar in structure (Duellman and Trueb, 1994).

Lipid glands will be mentioned first, as they appear to garner the least attention in the literature. They are, however, known to secrete lipid products that are thought to function in maintaining moisture levels (Duellman and Trueb, 1994).

Lipids, however, could actually serve a much larger role in chemical signaling than much of the amphibian literature has given them credit for (Alberts, 1990; Houck, 2009). Lipid glands are often (but not always) larger than granular glands, and are distributed relatively evenly across the body. They are present throughout the stratum spongiosum (Duellman and Trueb, 1994).

Mucous glands are the smallest of the three classes of integumental glands and are completely enclosed within the stratum spongiosum (Duellman and Trueb, 1994; Hecker et al., 2003). The mucous glands secrete mucopolysaccharides which function in coating the skin to maintain moisture. These glands are distributed abundantly and evenly across the body, though due to their small size they may seem less abundant than other types (Hecker et al., 2003). Mucous glands secrete constantly, and require no specific activation (Duellman and Trueb, 1994).

Granular (serous) glands are typically intermediate in size (Duellman and Trueb, 1994), though in some cases they are much larger than the surrounding glands (Hecker et al., 2003; personal observation). Granular glands exist mostly in the stratum spongiosum, though their bases can extend into the stratum compactum. These glands are known to secrete proteinaceous products, and require specific neural or humoral stimulation for secretion (Duellman and Trueb, 1994; Pool and Dent, 1977; Pool et al., 1977; Hoffman and Dent, 1977). In addition to proteins, granular glands are known to produce peptides, carbohydrates, biogenic amines, guanidine derivatives, steroids and alkaloids (Largen and Woodley, 2008).

Granular glands are less widely-distributed than mucous or lipid glands, and are more specific to the appropriate area of the body for their function (Duellman

and Trueb, 1994). These glands are known to be the secretory origin of amphibian toxins (when present), though Neuwirth (1979) concluded that this was secondarily-derived from a more primitive function. Other (non-toxin) granular gland secretions have been shown to be pheromones (Kiernan et al., 1990; Hecker et al., 2003; Largen and Woodley, 2008), and this seems highly likely to be the more primitive function that Neuwirth (1979) proposed. Interestingly, there seems to be a close relationship between mucous glands and granular glands; granular glands are thought to have transitioned from mucous glands, and intermediate forms are known to exist (Duellman and Trueb, 1994; Fontana et al., 2006).

Combinations of these glands are known to aggregate in various regions of the body to form large and obvious "macroglands" (Duellman and Trueb, 1994). These macroglands would be the equivalent of the Mental and Postcloacal Glands. Indeed, the Mental Gland of Plethodontids is one of the most characteristic accumulations of granular and mucous glands, which is hypertrophied in males seasonally (Sever, 1976b).

Granular glands can fluctuate in their appearance according to the season. During the breeding season, granular glands are cuboidal, which served to enlarge the lumen during this period. Endoplasmic reticulum (ER) and golgi apparatus activity is also heightened during this time. During the non-breeding season, secretory cells are columnar, which results in a reduced size of the lumen. ER activity is lessened and lipid and glycogen deposits can be observed. The hypertrophy of granular glands during the breeding season has been shown to be controlled by testosterone and prolactin (*Notopthalmus viridescens*, Pool and Dent,

1977). Gland hypertrophy has also been induced in female *Eurycea quadradigitata* (a Plethodontid) by experimental application of testosterone (Sever, 1976a).

Hecker et al., (2003) provided an excellent analysis of the glandular composition at the cellular level of the caudal integument of *Plethodon cinereus.* Mucous glands were shown to be evenly distributed (as expected) and made up ~20% of the total caudal glands. Histochemically, they were revealed to contain "basophilic, acid carbohydrates (probably sulfonated and carboxylated mucopolysaccharides)." A proteinaceous component was not observed (also as expected). This analysis also discovered two different types of granular (serous) glands in the tail skin; these different glands were termed S1 and S2 serous glands. The two types of serous glands were shown to have different biochemical composition of their secretory products (and it was on this basis that they were defined separately).

S1 serous glands were shown to make up an average of ~10% of the total caudal glands, but a higher proportion (20%) of the ventral portion of the tail. This number is slightly misleading, as S1 glands make up a much higher proportion of the total tissue mass in this region, because of their large size. These glands were up to five times the size of mucous glands in the same individuals. S1 glands were "characterized by an outer layer of simple cuboidal or columnar cells around a central lumen." S1 glands were shown to usually occupy the entire depth of the dermis (i.e. completely into the stratum compactum). Serous glands of the S1 type were found to be mainly distributed along the ventral portion of the tail (the Postcloacal Gland, as per Simons and Felgenhauer, 1992; Simons et al., 1994; 1999).

These glands could also be found along the dorsal and lateral portions of the tail, but were usually around the size of large mucous glands in these regions (i.e. 5x smaller than when on the ventral tail base). Interestingly, males had a higher average percentage of S1 glands to the total gland composition of the ventral tail base (~25%), while females had less S1 glands compared to other gland types of that region (~17%), though this result was not statistically significant.

S2 serous glands composed ~70% of the total glands. In the tail, they were found mainly along the dorsal portion, though they could also be found extensively on the lateral regions. These glands were roughly 2-3 times the size of mucous glands (smaller than S1 glands). These glands were "characterized by a layer of squamous cells surrounding a lumen." Both types of serous glands had distinct histochemical properties that differentiate the two.

S1 cells (surrounding the lumen) had distinct cytoplasm that contains a "mildly acidophilic, non-carbohydrate, slightly proteinaceous component." The S2 cell cytoplasm was found to offer little staining. The secretory granules (in the lumen) of the S2 cells were found to be 1.5-2 times as large as those in the lumen of the S1 glands. S2 granules were shown to be "acidophilic, non-carbohydrate or nonreactive carbohydrate" with a "strong proteinaceous component." S1 granules were shown to contain "mildly acidophilic, neutral carbohydrates, with a slight positive charge that probably comes from aproteinaceous component." However, there was shown to be proteinaceous component present, though it was less than that of the S2 glands.

Based on the histochemical identity of the different gland types, and the way in which they are anatomically positioned, it is safe to conclude that S1 serous glands are the glands used for the Postcloacal Press behavior and pheromonal scent-marking. Hecker et al. (2003) did in fact draw this same conclusion, and related it to the same finding of Staub and Paladin (1997) using another Plethodontid, *Aneides lugubris*. Hecker et al. (2003) also concluded that S2 glands are used mainly for nutrient storage and predator defense (through adhesive secretions released from these tail regions; Largen and Woodley, 2008).

Largen and Woodley (2008) also evaluated the biochemistry of gland secretions from various glandular regions of the body in *P. shermani*.* Interestingly, unlike in *P. cinereus* (Hecker et al., 2003), dorsal granular glands were shown to be three times larger than ventral tail glands in *P. shermani*. Dorsal tail glands were shown to be emptied when the animals were stimulated to release the noxious and adhesive secretions (by handling). Similarly, ventral tail regions were shown to be emptied after bouts of scent-marking. Noticeable differences between gland regions were observed through histological analysis, and these results were very similar to Hecker et al. (2003). Biochemical analysis of the secretions from the different tail regions revealed that these regions differed substantially in the composition of proteins released. This is the only study to my knowledge that has analyzed the secretions of caudal glands through protein biochemistry methods.

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* *P. shermani* is a previous sub-population of *P. jordani* that has recently been classified as its own species (Highton and Peabody, 2000; e.g. Rollman et al., 1999 vs. Houck, 2009). See Section I.A.8 for information on *P. shermani* pheromones.

I.A.8. BIOCHEMISTRY OF KNOWN PLETHODONTID PHEROMONES

The first amphibian pheromone to be molecularly identified was the female-attracting pheromone Sodefrin, a decapeptide from the abdominal gland of the red-bellied newt, *Cynops pyrrhogaster* (Kikuyama et al., 1995; 1999; 2005; Kikuyama and Toyoda, 1999). Since then, few others have been identified, though two of the most well-studied amphibian pheromones belong to an animal from the Plethodontid family: the red-legged salamander, *Plethodon shermani* (Houck, 2009). To this day, salamanders are the only vertebrate system in which pheromones that directly affect female receptivity (true courtship pheromones) have been identified (Houck et al., 2007a). All of the identified Plethodontid pheromones have come from the Mental Gland, and thus most are thought to function mainly in courtship.

The first biochemical analysis of Mental Gland secretions of *P. shermani* was done by Rollman et al. (1999). This study utilized High-Performance Liquid Chromatography (HPLC) to separate proteinaceous components of the secretion by the basis of their charge (anion-exchange). This analysis revealed the major component of the *P. shermani* Mental Gland: Plethodon Receptivity Factor (PRF). PRF is a 22 kDa protein of the Interleukin-6 family of Cytokine proteins. PRF is composed of ~200 amino acids, and was originally thought to be present in four distinct isoforms (Rollman et al., 1999; Feldhoff et al., 1999). The different isoforms are fairly well-conserved: there is a 90% homology of molecular weight and N-terminal amino acid sequences, with 16 polymorphic amino acid sites. Further research has since revealed that there are three isoforms of PRF (termed B, C1 and C2). The fourth suspected isoform shared a very similar charge to the three PRF

isoforms, and was thus termed "C3" (R.C. Feldhoff, pers. comm.). C3 has since been revealed to be an 18 kDa (non-PRF) protein, which has an unknown function.

The second major component was originally described as a ~10 kDa protein (Rollman et al., 1999). This protein was later shown to be a 7 kDa protein: Plethodon Modulating Factor (PMF; Houck et al., 2007a). PMF is a member of the Three-finger protein superfamily, which have a diverse range of functions, including snake neuro- and cardio- muscarinic toxins, as well as a variety of non-toxin functions (Palmer et al., 2007a; Houck et al., 2007a). PMF ranges from 77-86 amino acids, with 161 polymorphic sites and 35% sequence dissimilarity. Essentially the only conserved region of structure is a crucial eight cysteines that serve as the core of the "three-finger" structure. This high degree of structural dissimilarity has led PMF to be described only as "hypervariable" (Palmer et al., 2007a).

When experimentally applied to the nasolabial grooves of a female salamander during courtship trials, PRF is known to enhance female receptivity, and reduce courtship time. As males respond to the female and coordinate their behaviors accordingly, the application of PRF eventually leads to males depositing spermatophores more quickly. This same effect is shown to take place when the entire pheromone mixture is applied (Houck and Reagan, 1990; Houck et al., 1998; 2007a; Rollman et al., 1999). Interestingly, PMF actually has the opposite effect when applied by itself, and causes a longer mating time (Houck et al., 2007a). While it technically decreases courtship when applied by itself, PMF appears to have a sedative-like effect on the female that may begin to elucidate its function (Wirsig-Wiechmann et al., 2002; 2006).

The combined weight of the two main pheromone components PRF and PMF constitutes 85% of the total pheromone mixture (Houck et al., 2007a; Feldhoff et al., 1999). Of this 85%, there is a consistent 2:1 ratio (PMF:PRF) that has been observed over multiple animals and multiple years (Fontana et al., 2007; Feldhoff et al., 1999). Of the remaining 15%, the protein C3 makes up the majority (Feldhoff, pers. comm.).

PRF has been shown to be present only in the Mental Gland of Plethodontids, while low levels of PMF mRNA has also been located in the intestine, liver and kidneys of both males and females (Fontana et al., 2007). PRF and PMF expression are widely-distributed across the Plethodontidae (Fontana et al., 2007; Palmer et al., 2007a). There is also a third component that can be present in trace amounts in *P. shermani,* though its presence is highly variable. This pheromone is called Sodefrin-like Precursor Factor (SPF), and is thought to perhaps be an ancestral form of sex pheromone (Palmer et al., 2007b; Fontana et al., 2007). This component is thought to be replacing PRF through positive selection (Palmer et al., 2007b). SPF has a molecular weight of ~23 kDa (Palmer et al., 2007b), and is shown through behavioral experiments to increase female receptivity (similar to PRF), in *Desmognathus ocoee* (Houck et al., 2007b).

While a great deal of insight has been provided into the biochemistry, mechanism of action and the molecular evolution of Plethodontid courtship pheromones, many questions still remain unanswered and their specific functions remain largely speculative.

I.B. RESEARCH FOCUS

The overall goal of this research is to expand upon our current understanding of how variable series of information can be conveyed through a chemical medium. As outlined in the background information above, the pheromone signals of Plethodontid salamanders can convey a wide variety of information between animals, and facilitate an equally wide-range of sociobiological functions for these animals. There is an underlying logical conclusion to be drawn from this system, which will form the foundation of this research: in order for a pheromone signal to convey complex and variable series of information, the equivalent degree of variability must be inherent within the composition of the pheromone signal itself.

There is a great deal of plasticity involved in pheromone communication systems, ranging from: the biochemistry of the pheromones themselves, the physiological condition of the sender and receiver, the pheromone reception behaviors and neurobiology, as well as the spatial and temporal context of the signal. The experimentation of this thesis will involve mainly the biochemistry of the pheromone mixture, as so much variability lies within this realm.

In order to discuss the variability within pheromone signaling systems, the semantic distinction of "variable series of information" should be elaborated upon. To do so, an example within the aforementioned biology will be of use: the phenomenon of individual recognition. Plethodontids are known to be capable of differentiating between the signals of separate individuals: recognizing self vs. conspecifics (Tristram, 1977; Simon and Madison, 1984; Anthony, 1993), and familiar vs. novel individuals (Madison, 1975; McGavin, 1978; Jaeger, 1981). In

order to differentiate between the signals of different individuals, a high degree of variability within the pheromone signal will be needed; otherwise, the scent of all individuals would appear identical to the receiver. This requires a vast amount of structural plasticity within the pheromone mixture. Thus, individual identity would be considered a "variable series of information," in that the scents of various individuals differ from each other within the same single category of information. The size of the sender is an additional example of a variable series, and this information is particularly relevant to the phenomenon of female mate-choice.

The biochemical composition of the pheromone mixture can vary in several ways. The primary mechanism for plasticity is the sheer amount of pheromone present. In this way, the exact position between two polarities can be established within the overall gradient. This would be an example of a graded signal (*sensu* Wilson, 1975). However, as vertebrate pheromones are generally a mixture of many different molecules, the relative ratios between these molecules may confer different informational aspects to the receiver. This same concept also exists at a still smaller scale: many of the Plethodontid pheromones have been shown to exist in numerous isoforms (Rollman et al., 1999; 2000; Palmer et al., 2007a). The presence and/or absence of particular pheromones, as well as the relative ratios between pheromone isoforms, are likely to be of tremendous importance in conveying variable series of information and carrying out their specific functions to a differing degree.

The following research will explore these aspects of pheromone variability, mainly within the context of female mate-choice. The specific objectives for each experiment will be addressed in the corresponding experimental section (II.A-E).

II. EXPERIMENTATION

II.A. BEHAVIORAL EXPERIMENTS

II.A.1. BODY-WASHES AS A PHEROMONE SOURCE

Objective

The first behavioral experiment was designed as a pilot study to test the efficacy of obtaining pheromones for experimental use through a "body wash." This experiment would test if body-wash water would act as a sufficient pheromone source for conspecific recognition. Females are predicted to spend a greater amount of time associating with and investigating the body-wash water than the control.

Methods

Salamanders (*Plethodon cinereus*) were collected from College Woods in Durham, NH on 11/12/2007 (IACUC # 071005). Salamanders were housed in the lab (20°C; 14:10 L:D cycle) for two weeks while any remaining food could be passed through the gut to aid in identification of sex via candling (Gillette and Peterson, 2001). This also eliminated any contamination of the water with digestive material. A single male salamander (n=10) was placed into a small Petri dish (body-wash container; 5 cm diameter x 1 cm) containing 5mL distilled water. Males remained in the body-wash containers for one hour. During this time, the males were able to actively mark the territory (Jaeger, 1986; Simons et al., 1994; 1997), and any passive cues could also be released. These passive cues could be considered any other chemicals from the sender, not due to Postcloacal Press behavior, through which a receiving organism could infer species-specific information about the territory-holder. After an hour had passed, males were removed from the body-wash

containers and the water was used to test female response. Source males were size-matched to the focal female within 2mm Snout-Vent Length (SVL).

Gravid females (n=10) were tested in open plastic containers (14 x 19 x 10 cm) for their preference to either: (1) marked water, or (2) a blank control. The bottom of each side of the container was lined with paper towels, onto which the 5mL of water from the appropriate treatments were poured. The side to receive the experimental (marked/body-wash) water was randomized across all trials. Females were allowed to acclimate to the test chamber in an overturned habituation cup (6.5 cm diameter x 6.5 cm; Gabor and Jaeger, 1995; Jaeger et al., 2002) for five minutes. They were then observed with continuous focal sampling for five minute trials. The side on which the full torso of the female was present was recorded at all times ("Time in Proximity"), as well as the number of investigatory "Nose-Taps" (NT; Jaeger and Gergits, 1979; Jaeger, 1984). The time was not considered Time in Proximity if the female vertically exceeded the half-way mark of the side wall of the container. All testing began no earlier than 8:00 p.m. to ensure appropriate nocturnal activity. Both Time-in-Proximity and the average number of Nose Taps were analyzed with a two-tailed T-test assuming unequal variance.

Results

Females spent significantly greater Time in Proximity to the experimental body-wash water than to a blank control (Figure 1; two-tailed T-test; df=17; t=3.4; p=0.0035). Females also exhibited a greater number of Nose-Taps to the side containing body-wash water (Figure 2; df=9; t=3.4; p=0.0074).

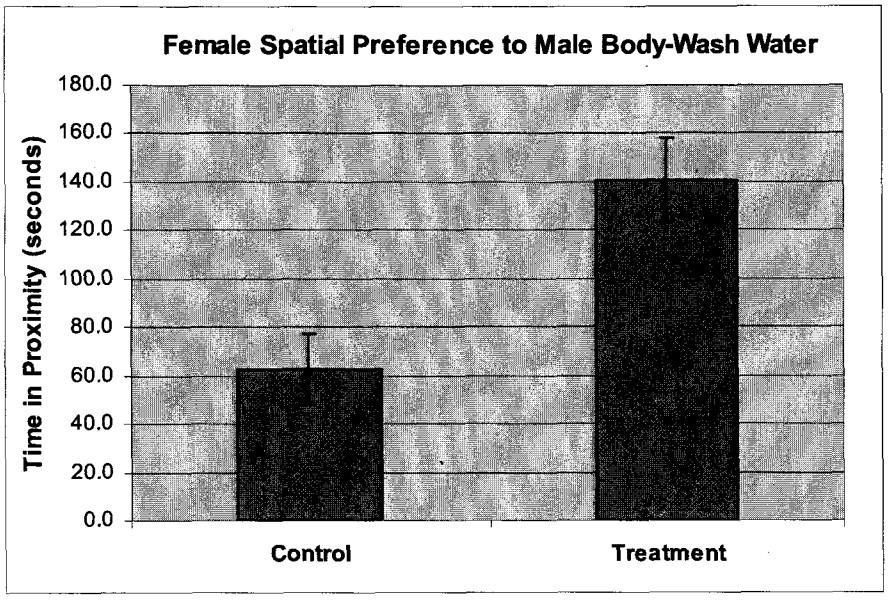

Figure 1. Time in Proximity of females to Body-Wash water versus a Blank Control. Females spent a significantly greater amount of time on the side containing water that had been marked by a male salamander (two-tailed T-test; p=0.0035). Error bars represent Standard Error.

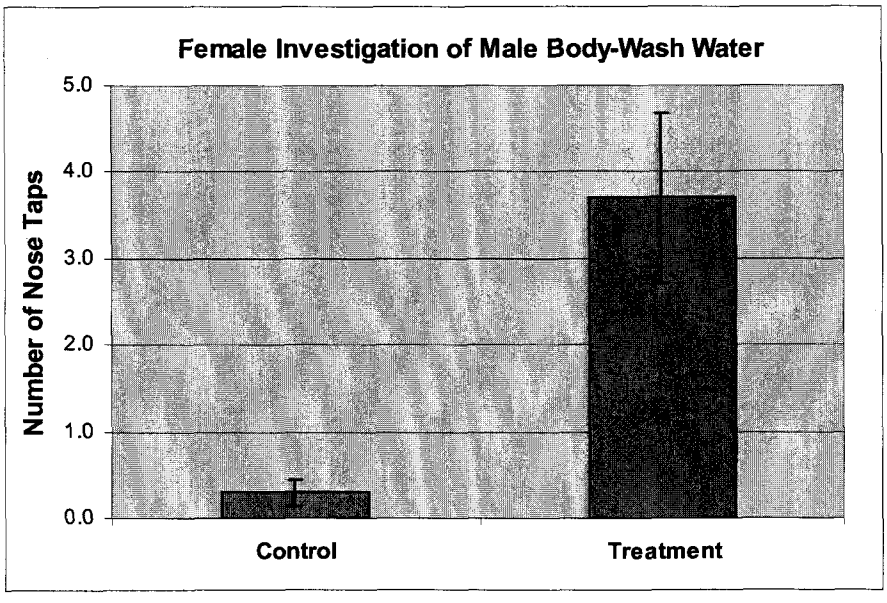

Figure 2. Number of Nose Taps of females to Body-Wash Water versus a Blank Control. Females exhibited a significantly greater number of Nose-Taps when the water had contained a male salamander for one hour (two-tailed T-test; p=0.0074). Error bars represent Standard Error.

Discussion

It is clear that gravid female salamanders were capable of differentiating between the two signals presented: a blank control versus the body-wash water of a conspecific male. Females demonstrated a strong and well-conserved preference to the scent of the conspecific male, spending significantly more Time in Proximity to the body wash signal as opposed to the unmarked side (Figure 1). Females also performed significantly more Nose Taps to the body-wash treatment (Figure 2). A very small number of Nose Taps took place at all to the blank control: only three females demonstrated one Nose Tap each to the unmarked treatment. This drastic difference in chemoinvestigatory behavior demonstrates a clear detection of pheromones in the body-wash water by the focal females. The behavioral preference of gravid females to associate with the scent of conspecific males has long been documented in *Plethodon cinereus* and other Plethodontids (see Jaeger, 1986; Dawley, 1998), however the exact source of the pheromones responsible for this discrimination have often been confounded through the experimental designs that have been utilized.

The purpose of this pilot study was to determine if the elimination of fecal pellets would still allow for the detection of male salamander odors by gravid females. No fecal pellets were present, as males were starved to allow for a complete clearance of the digestive tract; males were also only left in the body-wash containers for one hour. The null-hypothesis in this case is of course that females would not be able to detect anything, with the prediction being that there would be no significant preference to either treatment; this is not what was observed. Side-

bias was also ruled out because treatments were randomized across both sides evenly, yet the females elicited a strong preference specifically for the body-wash water. This implies that pheromones were present in the body-wash water, as indicated by the fact that the focal females were able to detect them.

Not only were gravid females able to detect salamander odors relative to an unmarked substrate, it seems as though they were *potentially* able to infer the sex of the sender through the odors presented. If the females had been exposed to intrasexual odors, it would be predicted that they should actively avoid them (i.e. Jaeger et al., 1986; Horne and Jaeger, 1988), whereas if females were exposed to male odors during the breeding season, it would be predicted that they would be attracted to these odors (Jaeger and Wise, 1991; Dawley, 1998). Females avoid male scents out of the breeding season (Jaeger and Gergits, 1979), but since the test was conducted only with gravid females during the breeding season, it seems likely that the females were attracted to the scent precisely because they were from males, thus indicating that they could infer this through the body-wash.

However, this conclusion may be premature when considering some inconsistencies in the literature regarding the degree to which females avoid female-marked substrates. Jaeger and Gergits (1979) did not find that females avoided intrasexual odors, while later research (Horne and Jaeger, 1988) found that females did avoid burrows marked by female conspecifics. The consensus seems to be that females are "less intimidated" by female scents than they are of male scents out of the breeding season (Dawley, 1998).

Similarly, some researchers claim that Plethodontid females will not be attracted to heterospecific male odors (Dawley, 1984b; 1986b; though again this was not originally shown by Jaeger and Gergits, 1979). This implies that the experimental females were likely able to specifically identify the source male odors as *P. cinereus*. However, since the determination of species was not explicitly tested in this experiment (i.e. the scent of a *P. cinereus* male versus that of a heterospecific presented simultaneously in a two-choice trial), this deduction should not be considered completely validated through these results alone. It is of course known that species information is conveyed through pheromone odors (see Section I.A.4), however these data merely imply that conspecific-recognition may be possible through a body-wash.

Testing for sex- and species-recognition, however, was not actually the objective of this preliminary study; instead, the objective was to verify that scent-marking would take place, and that a body-wash would act as an effective method for obtaining pheromones for further experimentation. Regarding this initial objective, these data confirm that body-wash water is a sufficient source of pheromones for the ability of gravid females to identify and gravitate towards the scent of conspecific males.

The use of a body-wash was intended to mimic a short-term territory that allows for active marking behaviors by the source male during its time within the container. It is known that males will perform the Postcloacal Press behavior to release scent-marks when placed on neutral (unmarked) substrates, even within short periods of time (Simons et al., 1997; 1999). Indeed, visual observations of the

body-wash often revealed cloudy secretions which are drastically different from fecal pellets. Obvious scent-marks were not seen in every case, but the sensitivity of the chemoreception mechanisms of these individuals is no doubt far greater than observations by the unaided human eye. The high degree of conservation of female behavior across all trials reveals that this is true; even in cases where no obvious scent-marks could be seen, the females as a whole acted as would be predicted if they were able to detect the scent-marks.

The body-wash water should contain scent-marking signals that have been actively deposited by the source animal, but other cues may also be passively released into the body-wash and used by receiving organisms to infer certain aspects of information. This experiment does not explicitly differentiate between these types of cues (active vs. passive), though based on the large volume of research into the use of scent-marks as territorial advertisements, it is clear that postcloacal odors (which are actively/voluntarily released; Jaeger, 1984; Simons et al., 1997; Hoffman and Dent, 1977; Pool and Dent, 1977; Pool et al., 1977) are responsible for conveying a great deal of information (see Section I.A.4/I.A.6).

In either case, the synthesis of all chemical signals released by the animal's integument will be present, and thus receivers should be able to infer all of the information that is encoded by the combination of these molecules. This fact provides additional support for the above interpretation that species and sex information is likely to have been inferred through the body-wash in this experiment. Ultimately, the only source of information that is possibly excluded is the fecal pellets themselves (see Section II.A.2 for more information on fecal pellets vs. integumental glands as the source of certain information).

Having established the feasibility of using body-washes as experimental pheromone sources, this method will thus be employed in the following studies to experiment more rigorously with the capabilities of this communication system.

II.A.2. FEMALE PREFERENCE FOR MALE DIET-QUALITY

Objective

This experiment was designed to test if females could establish a preference for males recently fed on a high-quality diet over those on a lower-quality diet, through the use of a body-wash. Previous research has concluded that females are able to infer information about the diet-quality of a territory-holder from information contained in fecal pellets, with males on a higher-quality diet being more desirable to females (Walls et al., 1989; Jaeger and Wise, 1991). However, the source of the pheromones responsible for this behavioral function is still unknown. The findings of Section II.A.1 concluded that a body-wash was sufficient for conspecific recognition*, while this experiment would then test if mate-assessment could also be facilitated in this manner.

This experiment would also serve to verify the findings of Walls et al. (1989) in the New Hampshire population, while under the housing and experimental conditions to be used for future research. This would also act as a control trial: (1) to ensure that female mate-choice for high-quality diets could be used as a behavioral assay of pheromone differences, and (2) that the chosen food sources designated for "high-quality" and "low-quality" diets would have the desired effect over a long term of differential feeding. It is predicted that females would preferentially associate with and investigate the scent of better fed males.

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* Conspecific recognition is being used in terms of the findings of Section II.A.1, in which gravid females were able to detect the presence of male salamander pheromones in the body-wash, versus a blank control in which no salamander had been present. This is in contrast to actually differentiating between multiple different types of species, which has been shown to be possible in Plethodontids (Dawley, 1984b; 1986; Ovaska and Davis, 1992) and in *P. cinereus* (Jaeger and Gergits, 1979; see Discussion of Section II.A.1 for more).

Methods

Salamanders (*Plethodon* cinereus) were collected from College Woods in Durham, NH (IACUC # 071005). The salamanders were housed in the lab (20°C; 14:10 L:D cycle) in clear plastic containers (14 x 19 x 10 cm) with paper towels and an inverted Petri dish (9 cm diameter x 1.5 cm) for cover. All salamanders were initially housed for two weeks to ensure passage of food through the gut for accuracy during sexing (via candling). This also eliminated the effect of having residual nutrition being derived from the field-diet at the beginning of experimentation. Twelve males were selected and arranged into pairs for differential feeding (n=6 pairs).

Since size is a very important factor contributing to female mate-choice (Mathis, 1991; Marco et al., 1998; Verrell, 1995), each pair was size-matched (+/- 1mm SVL; +/- 3mm TL) prior to differential feeding. Though the differences in size were very slight, half of the larger males were placed on high-quality diets, while the other half were placed on low-quality diets to further reduce the variable of size prior to feeding. All animals were also checked for parasites (also shown to be inferred through pheromones; Maksimowich and Mathis, 2001), and only those with no parasites were used in forming pairs. In theory, the only remaining variables were the direct genetic input to pheromone variability (random across treatments) and the treatments of high- and low-quality diet.

Based on previous research (Walls et al., 1989; Gabor and Jaeger, 1995), high-quality diets were considered food sources high in fat and protein, but also low in chitin, as it is considered to drastically reduce digestion efficiency. The food

source for the high-quality treatment (HQ) consisted of Enchytraeid white worms, while the food source for the low-quality treatment (LQ) consisted of *Drosophila melanogaster*. Both groups were fed to satiation once per week.

The male pairs were maintained on their designated diet from November, 2007 until testing in May, 2008. Testing had to take place in May to provide time for the diets to have an effect, but also to ensure that females were being tested in the breeding season, and thus motivated to make a distinct mate-choice. Females were collected from Kingman Farm in Durham, NH (IACUC # 080301) in May, 2008. The animals were housed in the lab at 20°C on a 14:10 L:D cycle (IACUC #080502). Males were not fed two weeks prior to testing in order to avoid any fecal contamination of the body-wash water (and therefore eliminate fecal pellet communication).

Because of the rigors of size-matching with a small number of available males, male diet-pairs were the limiting factor in sample size. To overcome this, each male was placed in a larger body wash (15 mL; 9 cm diameter x 1.5 cm) for a longer period of time (24 hours), and 4 focal females were tested (independently) for their preference between scents of each male pair. Females (n=24) were tested in a plastic Y-maze (See Appendix B for diagram). Each side of the Y-maze was covered with an appropriately-sized piece of paper towels. Body-wash water (5 mL) from each male was placed on either side of the divider. The side onto which the high- or low-quality signal was placed was randomized across all trials to eliminate any side-bias that can be encountered in two-choice testing. Females were introduced into the central chamber with a divider lowered (see diagram) to allow 5 minutes of

habituation to the apparatus. After 5 minutes, the divider was lifted and females were allowed to proceed. Trials also lasted 5 minutes, and timing began when a female had fully entered one of the chambers. Females were observed for "Time in Proximity" (defined in this case as a female's head and both arms entering a chamber), and the number of "Nose Taps" to the substrate of a given side. The first side entered was also recorded, to monitor the first choice of the female. All testing started no earlier than 8:00 p.m. to ensure appropriate nocturnal activity. The number of times the female entered the side of a certain scent as her first choice was analyzed via X^2 analysis. The Time-in-Proximity to each scent and the number of Nose Taps on each side was analyzed by a two-tailed T-test with unequal variance.

Results

Females made a statistically significant choice to enter the side with the scent of a high-quality male over the side with the scent of a low-quality male (17 vs. 7 respectively; $X^2=4.16$; $p<0.05$). Females also spent significantly more time in the chamber containing the scent of a high-quality male (Figure 3; two-tailed T-test; df=46; t=3.2; p=0.0028). Females also exhibited a significantly greater number of Nose Taps on the substrate containing the body-wash of the high-quality males than that of low-quality males (Figure 4; df=26; t=4.6; ; $p<0.0001$).

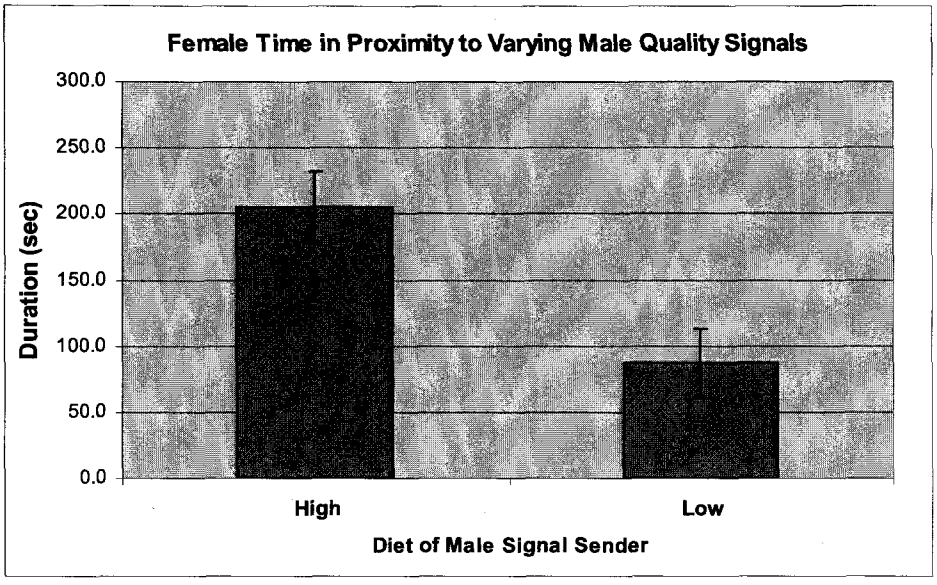

Figure 3. Average time spent by gravid females in the chamber containing body-wash water from males on high- and low-quality diets. Females spent significantly more time on the side with the high-quality scent (two-tailed T-test; p=0.0028). Error bars represent standard error.

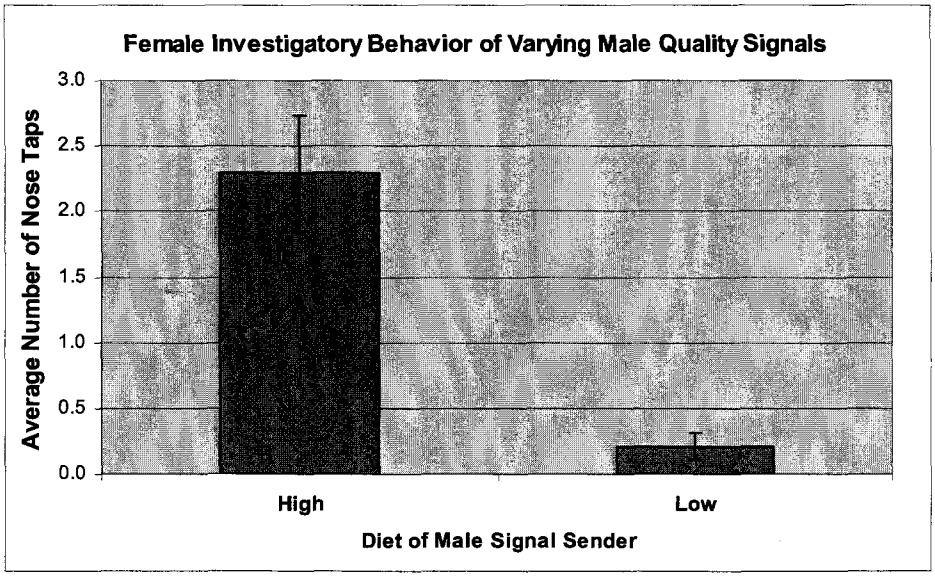

Figure 4. Mean number of Nose Taps by gravid females to the body-wash water containing the scent of high- or low-quality males. Females investigated the high-quality body-wash significantly more than low-quality males (two-tailed T-test; p<0.0001). Error bars represent Standard Error.

Discussion

Gravid female salamanders exhibited a strong spatial preference to associate with the scents of males kept on High-Quality diets (Figure 3). Females also exhibited more investigatory Nose Taps to the High-Quality scents (Figure 4). These experimental scents were obtained via body-wash, and therefore it seems that integumental signals themselves (rather than fecal cues) were responsible for facilitating this preference (see below).

When testing the ability of Plethodontids to discern specific characteristics of the source animal through a pheromone medium, most researchers have utilized either whole fecal pellets, or the substrate on which the sender has been allowed to inhabit (usually also therefore containing fecal pellets). This type of analysis allows for a great deal of insight into the capabilities of information transfer within the communication system, but it does not allow for specific insight into the source of the pheromones responsible. With this type of experimental approach, it remains unclear as to whether true pheromone signals are being actively produced by the sender, or if the receiver is simply using passive cues left behind by the individual.

For example, Walls et al. (1989) demonstrated the ability of gravid females to differentiate between High- and Low-Quality diets, but the source of the pheromones in question was the fecal pellets of the experimental males. Thus, it is unclear whether the males possess a molecule specifically designed to convey the quality of the diet, or if the females were merely utilizing other cues such as the composition of the fecal pellet itself (i.e. partially undigested food particles present therein). Walls et al. (1989) concluded that olfactory cues from the fecal pellets

could very well be used to assess its composition, but also that visual inspection of the proportion of chitinous components could also play a large role. Jaeger and Wise (1991) described a "fecal squashing" snout behavior unique to gravid females that presumably allows for the assessment of the appropriate cues, and this provided additional strong evidence for the case that fecal pellets were used in mate-assessment. Still, it remained unclear as to whether fecal pellets themselves are actively coated by pheromone scent-marks, or if there are other passive cues within the fecal pellets being utilized. It has often been stated that feces "contain" pheromones, but the issue of active signals vs. passive cues remained largely unaddressed in the earlier literature (except very briefly by Jaeger, 1986; see below).

Interestingly, much of the information shown to be inferred through fecal communication in Plethodontids is highly complex, such as the ability to recognize the scent of self vs. conspecifics (Tristram, 1977; Simon and Madison, 1984; Anthony, 1993), and familiar vs. novel individuals (Madison, 1975; McGavin, 1978; Jaeger, 1981). Both of these abilities require the identity of the sender to be encoded within the scent-marks themselves; this type of highly-complex information seems far too intricate to be facilitated by passive cues gained solely from the investigation of fecal pellets. Indeed, research by Simon and Madison (1984) tested the self-recognition ability of red-backed salamanders using both whole fecal pellets, as well as washes of the cloacal region*. This research concluded that both the cloacal region *and* fecal pellets themselves elicited the same efficacy in this ability. Since the fecal pellets contain the "cloacal" secretions (but not vice versa), it seems that active

* The "cloacal region" of Simon and Madison (1984) and Jaeger (1986) was later specified as the Postcloacal region; see Discussion in Simons and Felgenhauer (1992).

scent-marks of non-fecal origin are in fact the source of these complex pheromones, and "not a by-product of the chemical composition of the feces" (Jaeger, 1986).

The results of the present experiment, however, did not test for individual-recognition, which would require a higher-degree of structural variability in the responsible pheromone molecule(s) than would be found between mere fecal matter from different individuals. When investigating diet-quality (which is obviously highly related to the composition of the fecal pellets), it could easily be thought that cues from the pellets themselves could be providing adequate dietary information to the receiver.

This experiment did manipulate diet-quality, but the males were on different diets for a long period of time (six months), resulting in noticeable differences in body size. Also, source animals were starved to completely clear the gut, and body-washes (not fecal pellets) were used as the pheromone source. Still, females strongly preferred the scent of the larger males. Thus, the results of this experiment imply that the condition of size is in fact decoupled from the fecal cues themselves, and appears to be an intentional, active pheromone signal that is deposited (most likely through the Postcloacal region known to be responsible for scent-marking molecules). An important distinction to consider is that the source males were maintained on different diets for a long period of time; thus, the proximate mechanism by which females were able to discern differences in male quality may very well have merely been the size of the animals, and not the actual direct quality of the dietary items *per se.*

It is known that information about the size of the sender is encoded in Plethodontid pheromones (see Section I.A.4). At this point, it may be that size-information is the sole active signal produced by males to convey their quality, and that other bits of information garnered by females could be derived from passive cues; information such as diet-quality and parasite-load could potentially be derived from cues of non-intracellular origin of the sender. Conversely, it could be that these signals are actively produced as pheromones from the sender. This experiment provided some initial insight that ruled out passive fecal cues in the detection of diet-quality (though *indirectly*, through size-differences acquired while on differing energy-budgets; this distinction will be further investigated in Section II.A.3).

A last point of interest in this experiment was that the gravid females not only exhibited an ability to preferentially associate with and investigate the scents of High-Quality males, but also that they were able to make significant choices about which scent to choose first out of two Y-maze chambers. This implies that the females were not only able to discern the information about size, but that they were able to do it prior to investigating the non-volatile compounds via the nasolabial grooves and the VNO (by Nose Tapping). In the Y-maze utilized (see Appendix B), the scents of both source males were lowered from the plane upon which the female initially began the trial. The females did significantly choose to enter the side with the High-Quality scent, before they were ever able to assess the non-volatile components.

This provides strong evidence for some information of size to be conveyed through the volatile components of the pheromone mixture. Based on the complexity of the information-transfer in this communication system, as well as the complexity of the glandular secretions (see Section I.A.6 and I.A.7), there can be little doubt that a variety of signals are utilized for various purposes. In this way, it may be that volatile cues provide crude estimates of certain types of information, while non-volatile components (capable of containing more information) could provide more highly-detailed aspects. This would explain the Nose Tapping of the signals that still took place a great deal more to the High-Quality individuals. The volatile components indicated to the females that the High-Quality scent was "worth" investigating first, and the non-volatile components acquired thereafter provided more specific information. Thus, it could be that information about size is an aspect which is conveyed through volatile molecules over a longer range. It could certainly be seen that this would be of advantage not only in mate-assessment, but also in territorial advertisement: the odors would be more readily available to intruders over a broader active range (wafting evenly through the territory as opposed to the places only where the individual had actively scent-marked).

II.A.3. ENVIRONMENTAL INPUT TO PHEROMONES: TIME-ON-DIET

Objective

Two preliminary experiments (see above sections) concluded that placing a male red-backed salamander (*P. cinereus*) into a container with water would act as a sufficient source of pheromones for two important pheromone functions: (1) conspecific recognition, and (2) distinguishing size of source males (resulting from differing diets). In the case of the latter, this was in conjunction with previous findings showing the ability of Plethodontids to differentiate between the size of different males through the use of pheromones (Mathis, 1991; Marco et al., 1998; Verrell, 1995). In the above experiments, however, this effect was observed after several months on different diets, and the resulting female preference could have been made solely through the changes in size of the source animals over that time. Thus, it still remains unclear as to how quickly the information of nutritional condition can be conveyed through the pheromonal pathways, and whether this information itself is actively conveyed, or indirectly inferred by the receiver based on other characteristics (i.e. size of the sender).

Walls et al. (1989) demonstrated the ability of females to exhibit a preference for high-quality diets, with their choice based on the first fecal pellet to be produced by the two males of differing diets. It could certainly be that this information may have been obtained by visual inspection or non-pheromonal olfactory cues derived from undigested matter of the fecal pellet itself. It is also known, however, that fecal pellets are coated with pheromones (Mathis, 1990a; Simons et al., 1992; Simons and Madison, 1984; Ovaska and Davis, 1992; Jaeger, 1986; Jaeger et al., 1986; Horne and

Jaeger, 1988). In short, the mechanism through which gravid females are able to discern the quality of a source male's diet is unknown. While the methodology of Section II.A.2 eliminated the variable of fecal pellets, the source individuals were maintained on the appropriate diet for several months. It is therefore still unclear as to whether the decision of gravid females was proximately based indirectly on size or the quality of the diet itself.

This experiment was designed to test the time-course of intersexual diet-quality communication. It was hypothesized that if there was an immediate effect (discernable by female choice), that the information of diet-quality and size are decoupled, and conveyed through separate mechanisms. If the effect was not seen for a longer period of time, it would indicate that females base their information of diet-quality indirectly, by the size of the signal sender acquired through a higher-quality diet.

Methods

Salamanders were collected from Kingman Farm in Durham, NH (IACUC # 080301) in August, 2008. These animals were removed from 20m-diameter plots (n=10) for an ecological study. Animals were housed in the lab (20°C; 14:10 L:D cycle) in individual plastic containers (15 x 15 x 5cm) with moist paper towels as substrate. All animals from the field were starved for two weeks after capture to enable easier sex-determination (via candling), at which point they were measured for Snout-Vent Length (SVL; mm), Total Length (TL; mm) and weight (g). Males were arranged into size-matched pairs (+/- 2 mm SVL; 5 mm TL; 0.1 g).

Since all individuals came from multiple plots, females were assigned to male pairings so that they were never from the same home plot. This was necessary considering the evidence for pheromones conveying information about spatial location and prior association (Madison, 1975; McGavin, 1978; Jaeger, 1981). Thus, a female could never bias her choice for a male from her own plot over a less familiar male (more distant from her territory and/or home range). Technically, there could still be an effect of selecting for males closer to the female's original plot, though this factor was randomized across all trials.

Females (n=12) were tested in a two-choice scenario for their preference between the two males of her corresponding pair. The testing order of females (and thus the scents of the corresponding male pairs) was randomized each week. Testing for female preference was begun after three weeks of male starvation. This was done to reduce any residual effect of field-quality diet prior to experimental feeding and ensure that both males of a pair had a sufficient amount of time to pass any remaining food through their gut. The first week of female testing ("Week 0") began prior to differential male feeding. Weight differences between males were calculated, and this was used to evenly distribute the scents of the slightly larger males between the left and right side of the chambers to minimize any bias for a certain side of the chamber corresponding to the slight size differences. In this way, the average of differences between male weights was identical on both sides of the chamber for Week 0. Immediately after testing on Week 0, the male whose scent was associated with the least by the female was chosen to be placed on the "High-Quality" diet, while the male whose scent the focal female spent more time in

association with was chosen to be placed on the "Low-Quality" diet. This was done so that the following week the female would be required to change her preference from the original male (even if it were only a slight preference) as a result of the diet, instead of further expanding her already slight preference for the initial male. Each following week of testing, the sides of the male scents were reversed in the next trial.

Feeding of males was always done the day after testing. Males placed on "High-Quality" diets were fed 0.1g of *Enchytraeus* white worms, while "Low-Quality" males were fed 12-14 wingless ("feeder") *Drosophila melanogaster*. Female preference testing for the following week was then done 6 days later (one week after initial testing), so the males had time to feed and digest before the female would again be tested between their scents. This pattern of testing and feeding was set to be done until a significant behavioral preference was observed and repeated.

The experimental chamber was a plastic Y-maze (see diagram - Appendix B) with paper towels in each chamber as substrate. Each week, both source males of a pair were placed into a 5 mL body-wash dish (5 cm diameter x 1 cm) for six hours prior to testing. After six hours had passed, the males were removed and the water was placed into the appropriate side of the Y-maze. The focal female was placed into the entrance of the Y-maze, with the divider lowered to block her access to the remainder of the Y-maze. This allowed for a five-minute habituation period, after which the divider was raised and the female was allowed to proceed down the chamber to the scents of the source males.

A continuous focal sampling method was used to assess female behavioral preference. The "Latency" to make a choice was recorded; timing began when the divider was lifted and ended when a female had entered into one of the chambers of the Y-maze. This tested how quickly the females responded to the male scents each week. In all cases, entering and leaving a chamber was considered having the head and both arms over the edge of the chamber in the appropriate direction (into the chamber for entering, and out of the chamber for leaving). Other recorded data included "Time-in-Proximity" and the number of "Nose Taps" (NT) to each side of the chamber. Time-in-Proximity was scored as the duration of time inside each chamber. Nose Taps were only counted when they were administered to the substrate ("marked" paper), not the sides of the chamber. The number of Nose Taps was also used to determine "Investigatory Rate" (NT/min) by dividing the number of Nose Taps by the number of seconds spent on a given side and converting into minutes. This normalizes the data according to the amount of time actually spent on a given side, since not all females spent the same amount of time on each side. Thus, the *rate* at which they investigated the substrate can still be directly compared.

Trials lasted for five minutes, and all testing took place starting at 8:00 p.m. to ensure appropriate nocturnal activity. Average Time-in-Proximity, Nose Taps and Investigatory Rate (NT/min) were analyzed with a two-tailed T-test (with unequal variance). "Latency" was analyzed with a Paired Wilcoxon-Signed Ranks Test. The number of Nose Taps to each scent were also analyzed via X^2 Analysis.

Results

When males were originally size-matched and starved for three weeks prior to differential feeding (Week 0), females exhibited no significant preference in the Time-in-Proximity (Figure 5) to the body-wash scent of either male (two-tailed T-test; df=18; t=0.1; p=0.91). Consequently, this means that females exhibited no noticeable side bias to either chamber of the Y-maze. After one week of feeding (Week 1), the females spent significantly greater Time-in-Proximity to the males that had been placed on High-Quality diets (df=22; t=2.6; p=0.017). The same trend was observed after another week of differential feeding (Week 2; df=22; t=2.4; p=0.027), after the scents of all the source males were placed on opposite sides as the week before.

Similarly, females did not perform a significantly greater number of Nose Taps (Figure 6) on average to either scent on Week 0 (two-tailed T-test; df=10; t=0.7; p=0.46), or even after one week of feeding (df=12; t=1.6; p=0.12). After two weeks of feeding, however, females did Nose Tap a significantly greater number of times (p=0.003). When the total number of Nose Taps (not the average) were analyzed via X^2 analysis, there was no significant difference in the number of Nose Taps at Week 0 (Left=6, Right=2; df=1; X^2=2; p>0.05). On Week 1, there was a significantly greater number of Nose Taps performed to the scent of High-Quality males (NT=12) versus Low-Quality males (NT=1; df=1; X^2=9.3; p<0.01) than would be expected at random.

When compensating for the amount of time spent on each side ("Investigatory Rate"; Figure 7), females did not exhibit more Nose Taps/minute to the scent of either male on Week 0 (two-tailed T-test; df=3; t=0.9; p=0.42); the same was true on Week 1 (df=11; t=0.8; p=0.42). However, females did investigate the scents of High-Quality males at a significantly greater rate than that of Low-Quality males after two weeks of differential feeding (Week 2; df=10; t=2.7; p=0.022).

Prior to differential feeding, when animals had been starved (Week 0), females took a significantly greater amount of time to make a behavioral choice, than when males had been fed for one week (Wilcoxon Signed-Ranks test; n=10, T=7; $p<0.02$). In other words, females were significantly quicker to respond with a behavioral choice when the source males had been fed ("Latency"; Figure 8). There was no significant difference between Week 1 and Week 2 (n=11; T=20; $p>0.05$).

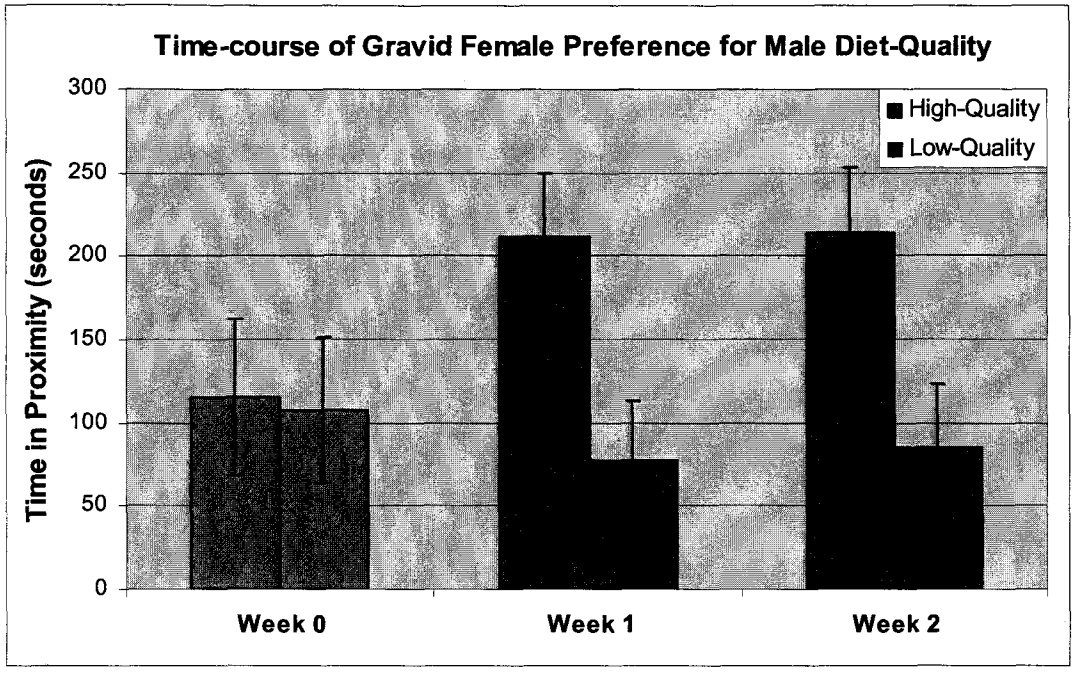

Figure 5. Average duration (+/- Standard Error) that gravid females (n=12) spent in association with the scents of males on High- vs. Low-Quality diets over the course of two weeks of differential feeding. Grey bars (Week 0) represent scents placed on the left and right sides of the experimental chamber respectively. Gravid females made no significant preference ($p>0.05$) in the first week (prior to feeding), but spent significantly more Time-in-Proximity to the scents of males on High-Quality diets within one week of differential feeding ($p=0.017$). The same preference was observed after two weeks of feeding ($p=0.027$).

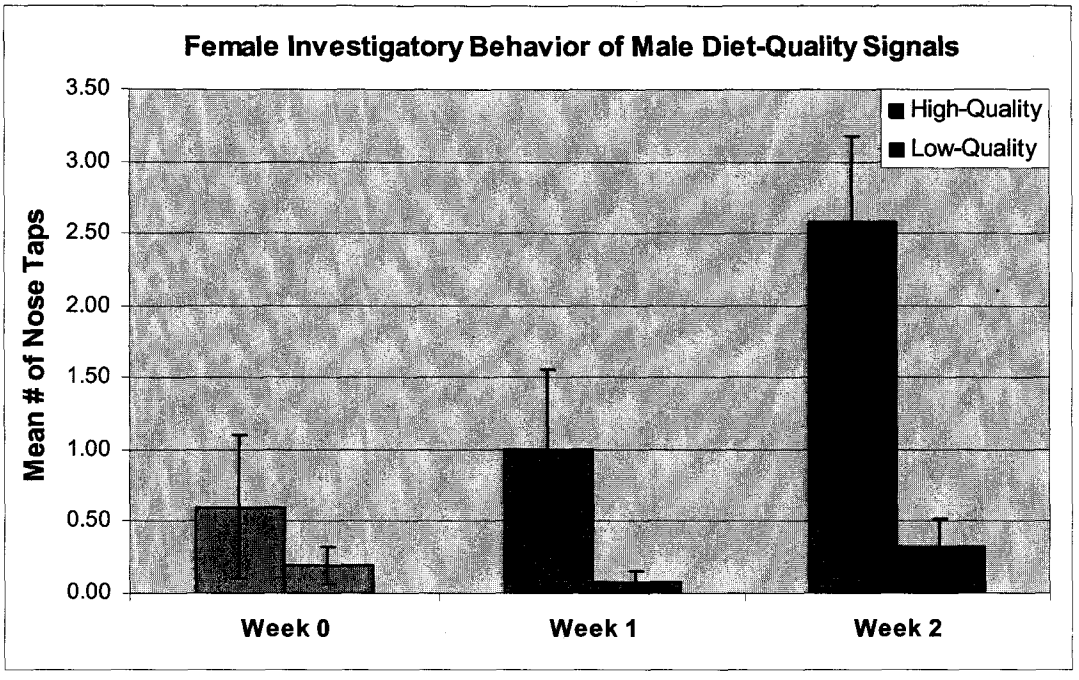

Figure 6. Average number of Nose Taps (+/- Standard Error) by gravid females (n=12) to the scents of males on High- and Low-Quality diets. Grey bars (Week 0) represent scents placed on the left and right sides of the experimental chamber respectively. Females did not perform a significantly greater number of Nose Taps ($p>0.05$) when the males were starved for three weeks, size-matched, and not on different diets (Week 0). There was also no significant difference after one week of differential feeding ($p>0.05$). After two weeks of feeding, females performed a significantly greater number of Nose Taps ($p=0.003$).

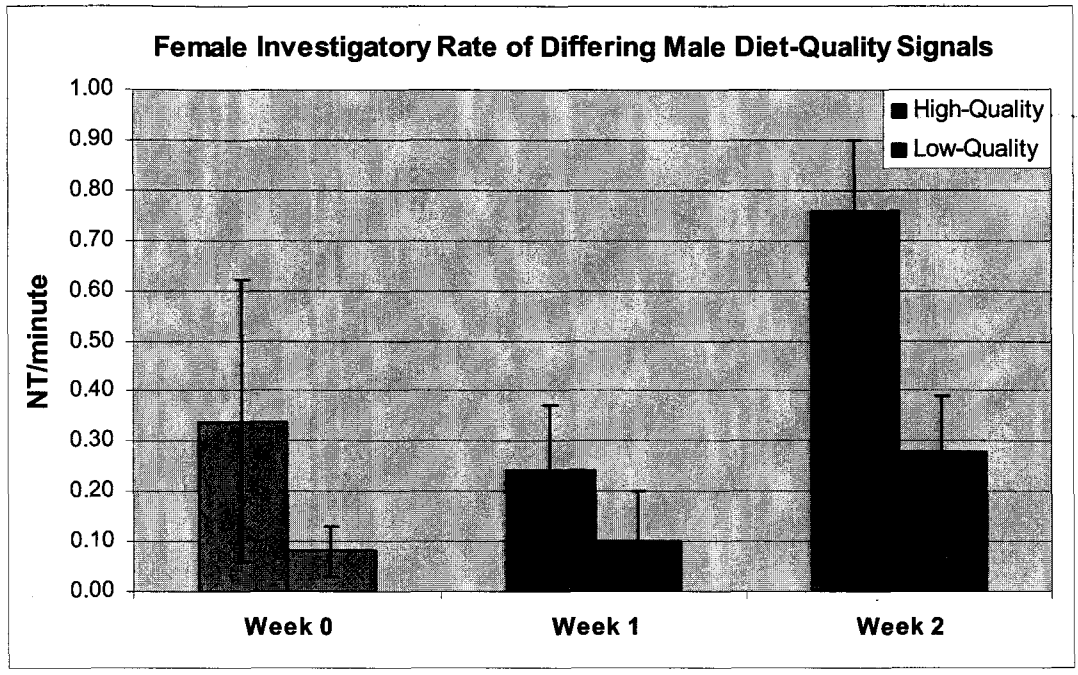

Figure 7. Average Investigatory Rate (NT/min; +/- Standard Error) of gravid females (n=12) to the scents of males on High- vs. Low-Quality diets. Grey bars (Week 0) represent scents placed on the left and right sides of the experimental chamber respectively. Females did not investigate either signal at a significantly higher rate when the males were starved for three weeks, size-matched, and on the same diet (Week 0; $p>0.05$). There was also no significant difference after one week of feeding ($p>0.05$). After two weeks of feeding, however, females did investigate the scent of High-Quality males at a significantly faster rate ($p=0.022$).

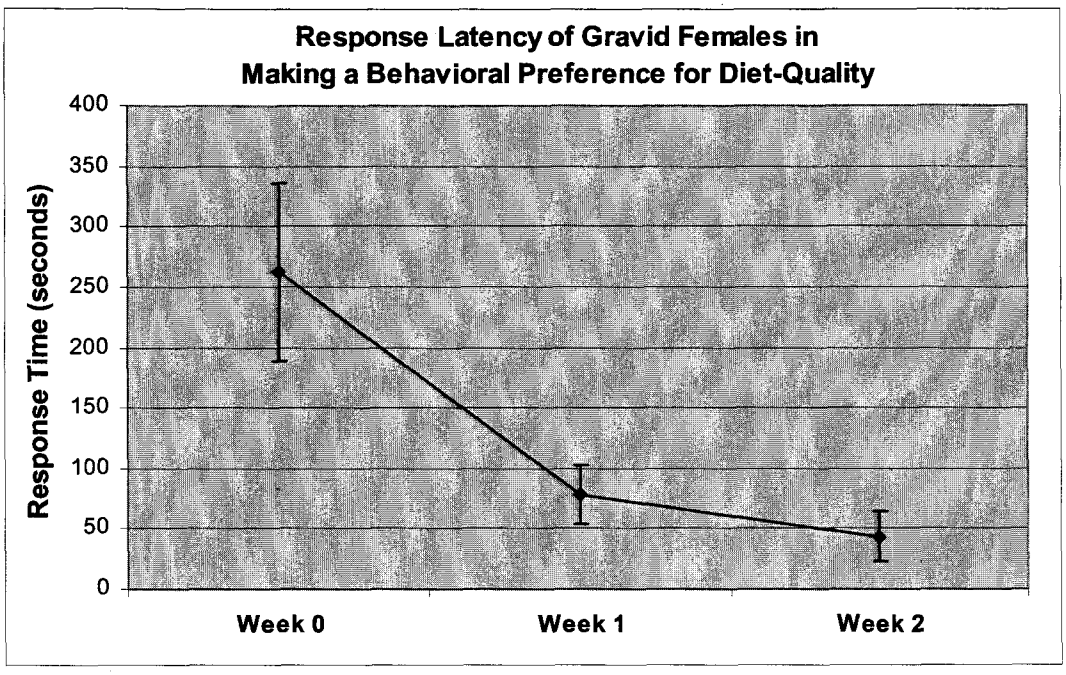

Figure 8. Average response latency (+/- Standard Error) of gravid females (n=12) in making a behavioral choice after three weeks of starvation ("Week 0"), and again after one and two weeks that the source males had been on their corresponding diets. The behavioral choice (seen in Figure 5) took significantly less time (n=10; T=7; $p<0.02$) after one week of feeding (Week 1) than when males had been starved for three weeks, size-matched, and not on different diets (Week 0).

Discussion

For the first week of testing female preference (Week 0), both males of each pair were on Field-Quality diets and had been starved for three weeks to ensure a minimal effect of the residual dietary cues. At this point, females made no significant preference to differentially associate with (Figure 5), or investigate (Figure 6 and 7) the scent of either male. In other words, there was no clear behavioral preference when the males were of equal, field-quality, condition. Remarkably, even after one week of differential feeding, gravid females spent significantly more time associating with the scent of High-Quality males (Figure 5). This same trend was repeated almost identically on the following week, after an additional week of differential feeding.

After one week of feeding, there is some ambiguity in the Nose Tap data (Figure 6). As expected, there was no difference in Nose Tapping on Week 0. When analyzing the data for Week 1 via X^2 analysis, there was a significantly greater number of Nose Taps to the High-Quality scents (12) as opposed to the Low-Quality scents (1). However, when analyzed with a two-tailed T-test assuming unequal variance, this was not significant, nor was the investigatory rate data (NT/min; Figure 7). This lack of significance in Nose Tapping on Week 1 is most likely because all twelve High-Quality Nose Taps were distributed across only five individuals (even though only one female did a single NT to the Low-Quality scent). Regardless, after two weeks of feeding, gravid females did Nose Tap the High-Quality signals significantly more (Figure 6), and also at a significantly faster rate (Figure 7), as analyzed by the two-tailed T-test. Even if the females had not investigated the two

scents differently (which they ultimately did after two weeks, and even week one looks convincing considering the low numbers of Nose Taps and the X^2 statistic), there was still a clear spatial preference to the scent of High-Quality males, after only one week of differential feeding.

Thus, gravid females are able to distinguish between the quality of the diet of the male sender, and more importantly, this information is conveyed directly and over a short period of time. In other words, the detection of diet-quality by receivers does not appear to be based solely upon passive fecal cues. It is also shown that diet-quality is not indirectly inferred through the information of size (as the discussion from Section II.A.2 implied may be the case).

Not only were gravid females able to make strong preferences for High-Quality males, they also made their choices significantly faster (Figure 8) when the animals had been fed (Week 1) rather than starved (Week 0). The response latency of females in making their behavioral preference was long when both males were on the same diets (Field-Quality), and when they had been starved for three weeks. The response latency was reduced dramatically when the animals had been fed, and placed on differing diets. Several factors are likely to be at play here. For one, it could be that the scents of both male parties were much stronger after having been fed, and thus the females were quicker to respond to them. Secondly, the behavioral choice of which male to associate with may have been much clearer to the females after the males were on such different diets, as opposed to when they were both similarly fed. In other words, when both males were equally starved and on equal diets (on average), the choice of which male to associate with may not have been as

one-sided. However, when the males had been placed on such different quality diets, it may have been much clearer as to which animal the female should associate with, and thus the females responded more quickly.

As in the experiment above (Section II.A.2), the females were tested in a raised Y-maze, and thus volatile scents were likely to be responsible for facilitating at least a portion of the behavioral choice of the females. This is particularly true in the case of the changing response latencies after feeding had taken place. The latency ended when the female first chose a given chamber, but at that point they had yet to be exposed to the non-volatile, substrate-borne signals of either male. Yet, females did respond much more quickly after male feeding had taken place, and thus it is clear that some aspect of this information was obtained through volatile cues. The findings of Section II.A.2 implied that size (or potentially even the diet-quality directly) was being conveyed through volatile cues, and the current experiment lends even more support to the idea that the quality of the diet (perhaps *along with* the size of the sender) could be conveyed through a volatile medium.

Based on the predictions of chemical signaling (see Alberts, 1992; Wilson, 1975; Section I.A.6), non-volatile molecules are larger, which have a greater potential for structural variability, and thus a greater potential for information-storage. Therefore, the overall communication of these volatile male quality-indicators may be a simpler situation. It could be that there is simply a volatile salamander odor, the volume of which conveys the amount of male scent present; if this were an honest (energy-dependent) signal, then the greater amount of scent present would indicate a more "desirable" male with which to associate (one on a

higher energy-budget). This idea gains some support from the difference in response latencies (Figure 8) of gravid females between Week 0 (after starvation) and Week 1 (after feeding). This is also not in contradiction to the findings of Section II.A.2, which suggested that perhaps size was the key information being conveyed, and that diet-quality was only indirectly inferred. In the case of that experiment, diet-quality was obviously a large factor, as noticeable size differences were observed between the males after six months of differential feeding: the two treatment groups had drastically different energy-stores with which to invest into pheromone signaling. Therefore, it may be that size and energy are not actually decoupled in the case of volatile odors. Even still, there could be multiple chemicals conveying these different characteristics, and functioning as a composite signal (*sensu* Wilson, 1975).

The differences in Nose Tapping investigatory behavior seemed to lag behind the spatial preferences of females to associate with High-Quality male scents. Again, this is likely a statistical artifact due to the low number of individuals that exhibited any Nose Taps on Week 1. Even if it isn't, there were strong differences after two weeks of feeding (Figures 6 and 7). Not only were more Nose Taps exhibited to High-Quality males after two weeks of feeding (as females spent more time in proximity to those signals), but the females also Nose Tapped High-Quality scents at a significantly faster rate as well (when compensated for the amount of time the female spent on a given side). Again, statistically significant differences were not noticed until two weeks of feeding, but this is still a surprisingly short period of time in which to differentiate between diet-qualities of the sender. The non-volatile

components appear to also have a large role in assessing this information and facilitating aspects of this phenomenon; if this were not so, it would not be expected that females would differ in their investigatory rates.

Though these data are fairly conclusive, some limitations of the experimental design should be considered. For one, after each week the side to receive the scent of each male was alternated, so that females were not simply choosing the same side each week. Also, the male chosen to be placed on the High-Quality diet was deemed the individual with which the female associated the least on Week 0 (in an attempt to cause her to override even a slight preference for the other individual, as a result of diet, and not to expand upon an already slight preference for that individual). Thus, for Week 1, some females may have chosen to associate with the same side, and not the individual *per se*. However, this effect is cancelled out by the fact that on the following week (Week 2) the scents of each male were again reversed, and still the females demonstrated an almost identical degree of preference, but would had to have chosen a different side. In addition, *P. cinereus* is capable of distinguishing the identities of different individuals, and recognizing familiar vs. novel individuals (Tristram, 1977; Simon and Madison, 1984; Anthony, 1993; Madison, 1975; McGavin, 1978; Jaeger, 1981); thus, the females could not have been "guessing" as to which male was the one with which she associated in previous trials.

Even considering this potential limitation, it seems clear that gravid females can differentiate between male diet-qualities after only one week of differential feeding. Diet-quality also appears to be directly communicated by the sender, not indirectly inferred through the information of size.

II.B. PRELIMINARY BIOCHEMICAL ANALYSIS

Objective

While a substantial amount of work has elucidated several behavioral functions of Plethodontid pheromones, little is known about the biochemical nature of these pheromone mixtures. Of the specific biochemical analyses that have been done, a large proportion of it has been with the submandibular Mental Gland of the red-legged salamander, *Plethodon shermani* (Rollman et al., 1999; Feldhoff et al., 1999; Houck et al. 2007a). In *P. cinereus*, the secretory products of various glandular regions have been roughly categorized through histological analysis (Section I.A.7), though the specific nature of these chemicals remains unclear. The objective of this section was to analyze the pheromone extracts of the *P. cinereus* Mental (MG) and Postcloacal Gland (PCG) using the same methods that have been applied to investigate the Mental Gland of *P. shermani*. These methods include Reverse-Phase High Performance Liquid Chromatography (RP-HPLC; II.B.1.) and Sodium-Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE; II.B.2.).

II.B.1. RP-HPLC ANALYSIS OF PHEROMONE COMPONENTS (MG + PCG)

Methods

Salamanders were collected from Kingman Farm in Durham, NH in May of 2008 (IACUC #080301) and housed in the lab at 20°C on a 14:10 L:D cycle (IACUC #080502). Animals were starved for two weeks after capture to enable the determination of sex via candling. 10 male salamanders were identified for surgical removal of the Mental Gland (MG) and the Postcloacal Gland (PCG; also Ventral Tail Base - VTB). Animals were anesthetized in 100mL of a 7% aqueous diethyl ether

solution for 8-10 minutes. Anesthetization was concluded when an animal had lost its "righting-response," the ability to turn itself over when placed upon its back.

Once anesthetized, animals had their Mental and Postcloacal Glands surgically excised under the dissecting scope, with micro-forceps and scissors. The Mental Gland was removed by snipping a line through the skin in the center of the mentum (at the posterior base of the mandible), continuing to cut the skin along the distal edge of the mandible. In this way, the entire region of dermis within the mandibular region was removed. For Postcloacal Glands (PCG/VTB), the skin was removed by initially making an incision 1mm posterior to the cloaca, extending across the width of the tail that touches the substrate in the normal resting posture. Another incision of equal width was made 1.5 cm posterior to the cloaca (to normalize the volume of skin taken across all animals), with lengthwise incisions connecting the initial incisions. The entire region was then underscored with the scissors; the skin was then removed, revealing the underlying caudal musculature.

The MG region of interest was identified through research of the appropriate glandular location (Pool and Dent, 1977; Sever, 1976b; Simons and Felgenhauer, 1992; Duellman and Trueb, 1994; Rollman et al., 1999; Houck et al., 2007a), personal communication (Houck, Kiemnec and Feldhoff), as well as personal observation under a dissecting microscope. The PCG region of interest was also identified through research of the glandular areas (Simons and Felgenhauer, 1992; Simons et al., 1994; 1999; Duellman and Trueb, 1994; Hecker et al, 2003; Largen and Woodley, 2008), as well as through personal observation under dissecting microscopes. The S1 serous glands (Hecker et al., 2003) of the ventral tail base are

visibly different in morphology than the surrounding lipid and mucous glands (under low-magnification microscopic observation). The S1 serous glands of the PCG were later verified through histological analysis (Section II.E).

In both cases, immediately after removing the glandular region, the skin was placed into solution for protein extraction. The extraction solution consisted of 0.8 mM acetylcholine-chloride (AchCl) in amphibian Ringer's solution (phosphate buffered saline; PBS). AchCl was previously shown to cause release of granular gland secretions (Hoffman and Dent, 1977). Glands were placed in 200 μL of extraction solution (in 1.5 mL microfuge tubes) for 60 minutes, after which the skin was removed. The AchCl/PBS was then centrifuged (14,000xG) at 4°C for 10 minutes; the supernatant was removed, placed into a clean microfuge tube and re-centrifuged under the same conditions for another 10 minutes. This process was repeated one additional time (a total of three rounds of centrifugation), after which the supernatant was removed, placed in a clean microfuge tube and frozen at -20°C.

Prior to the analysis of the salamanders collected from Kingman Farm (Durham, NH), a preliminary analysis was done on frozen samples of a *P. cinereus* MG from a population in Virginia, near the field site of Dr. Houck and Dr. Feldhoff (Highlands, North Carolina). This sample had been surgically removed in 2002 (by Dr. Lynne Houck), underwent the same extraction protocol described above (by Dr. Richard Feldhoff), frozen at -80°C, but never biochemically analyzed until September, 2008 after discussion of this project (Figure 9; see HPLC method below).

Samples from the NH population (surgically removed in November, 2008) were then sent on dry ice to the lab of Dr. Richard Feldhoff at the University of Louisville (Louisville, KY) for RP-HPLC analysis (Rollman et al., 2000; R.C. Feldhoff, pers. comm.) in March, 2009. Samples (n=10 individuals; 2 glands/individual) were thawed and loaded into a C18 RP-HPLC column (5 μm; 4.6 x 150 mm; Vydac, Hesperia, California) that had been equilibrated with Buffer A (0.1% trifluoroacetic acid; TFA). The column was then eluted with a linear gradient of Buffer B (70% acetonitrile/0.08% TFA) at 1mL/minute for 70 minutes. The elution gradient is an increase in the ratio of Buffer B:A over the course of the run (with a 1.4% increase in Buffer B every minute). Absorbance was detected at a wavelength of 220 nm.

In the case of both the MG and the PCG, samples were analyzed individually (100 μL; from the ~200 μL total for each individual), but also as a pooled sample (as a 100 μL sample, from a combined pool of the remaining ~100 μL from each individual). 100 μL of the pool (for both the MG and the PCG) were run in duplicate, whereas individuals were run once (because the remainder had been used to form the pooled sample).

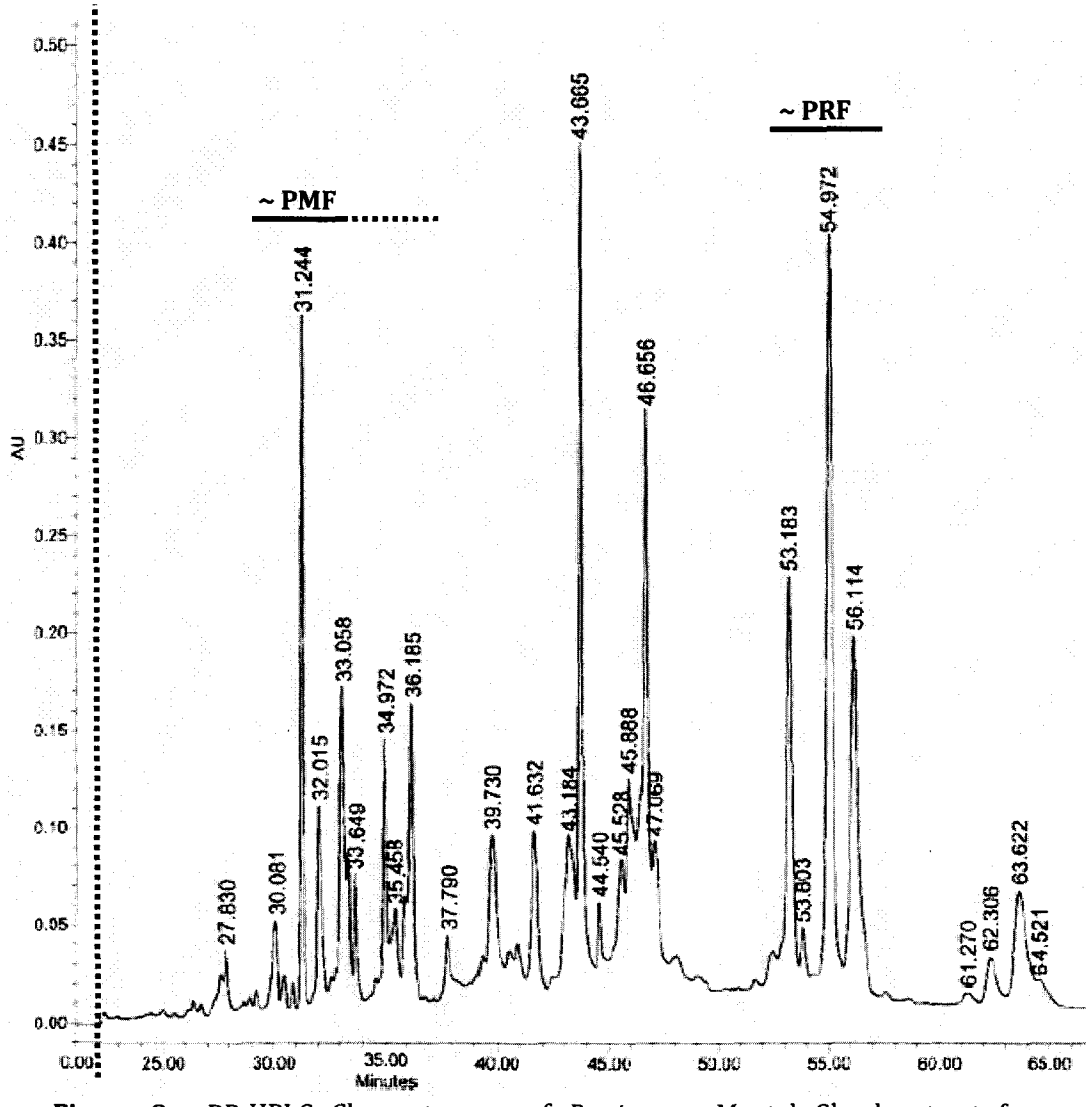

Figure 9. RP-HPLC Chromatogram of *P. cinereus* Mental Gland extract from a population in Virginia, USA. The independent variable is the elution time (minutes) of the various molecules, while the dependent variable is 200 nm absorption units (AU), representing a relative amount of molecules eluting at that time. The elution time (from 1-22 minutes) has been cut (vertical dashed line), as no molecules eluted earlier than ~26 minutes. There are numerous well-defined peaks in the known range of PRF (~53-56 above), and also a predominant spike in the known range of PMF (~31 above). The dashed line above the later eluents in the PMF range demonstrates that the exact elution boundary of PMF isoform variants is unknown.

The pooled sample of *P. cinereus* MG extract from the Virginia population demonstrated a number of molecules present, ranging in elution times from ~27 to ~65 minutes (Figure 9). The peaks also varied substantially in the number of absorbance units (AU; y-axis), indicating their relative differences in proportion (in volume) within the MG. Of these peaks, the most dominant peaks (AU $\geq$ 20) occurred at the following elution times: 31.24, 43.67, 46.66, 53.18, 54.97 and 56.11 (minutes; x-axis).

When comparing the pooled MG sample from the NH population (see Appendix A.2 for chromatograms), there was a lower number of peaks overall in the NH sample than the Virginia sample. The MG pool of the NH population was also far less resolved (the molecules were less separated from each other; i.e. less sharply-defined peaks). There was, however, still some overlap in the elution times of the dominant peaks. The most dominant peaks of the NH sample occurred at the following elution times: ~43.7, ~46.3, ~47-48, as well as two later peaks that occurred at ~58.7 and ~60.6 minutes.

When observing the chromatograms for the MG extracts from individual animals, there is a fair amount of variation in the number of peaks, as well as the elution time for the various peaks (see Appendix A.3 for chromatograms). That being said, there were still numerous peaks that were well-conserved across the individual runs (Figure 10). Even in the case of well-conserved peaks, there was a great deal of variability in the absorbance values (volume of the molecules eluting at that time) and the relative volume ratios between peaks.

The pooled PCG sample (the only one of which was from the NH population; see Appendix A.4) demonstrated a number of peaks ranging in retention time from ~5 to ~65 minutes. While there were numerous peaks observed, the resolution between peaks was poor. The most dominant peaks present in the pooled PCG sample were at the following elution times: ~14, ~34, ~40, numerous peaks within the ranges of ~43-49, ~56, ~58, ~60 minutes.

Upon observing individual PCG runs (see Appendix A.5), there were a number of well-conserved peaks across all individuals (Figure 11). As in the case of the Mental Gland (Figure 10), there was a great deal of variability in the relative ratios of the individual peaks, even when they were well-conserved in terms of which peaks were present.

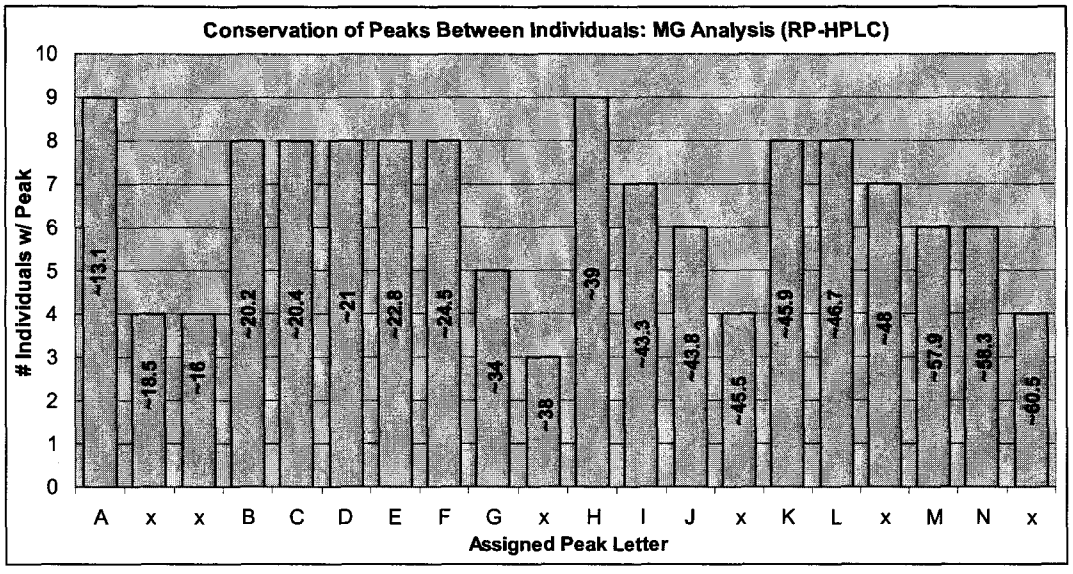

Figure 10. Histogram of the number of individuals that share corresponding peaks after RP-HPLC analysis of Mental Glands from individual animals. Peak letters were assigned based on absolute and relative retention times of each peak, as well as characteristic visual patterns of the specific peaks (see Appendix A.3 for chromatograms of individual runs with corresponding peak letters for each individual). Retention times (minutes) of the individual peaks are displayed numerically within the bar for each peak. Capital letters (A-N) correspond to consistent and obvious peak duplications in the various individual runs, while a lowercase "x" corresponds to more variable and less consistently identifiable peaks. There is no graphical representation in this figure of absorbance units of each peak (the relative volumes of the molecules at any given elution/retention time).

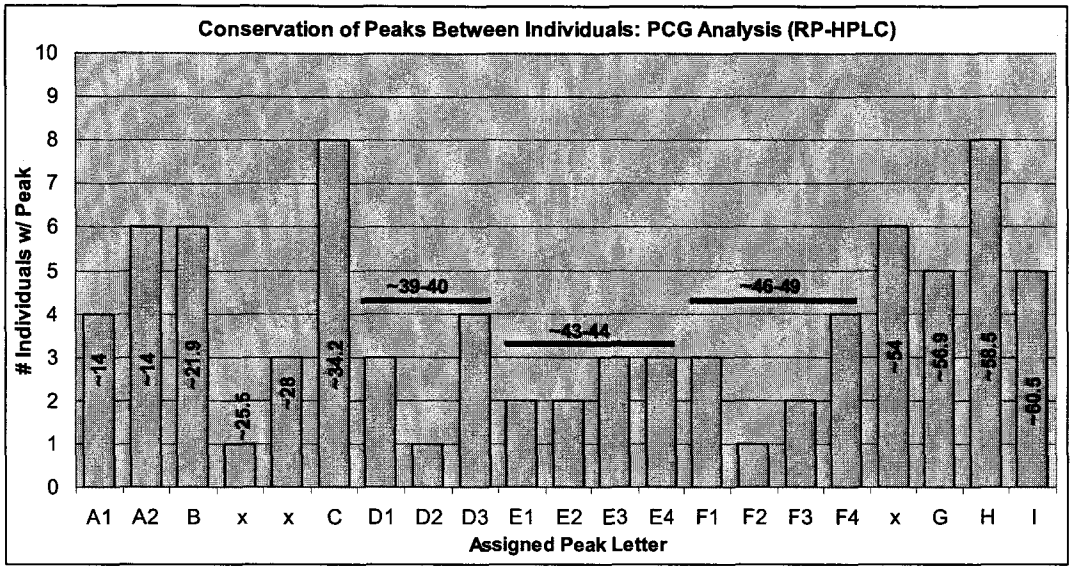

Figure 11. Histogram of the number of individuals that share corresponding peaks after RP-HPLC analysis of Postcloacal Glands from individual animals (see Appendix A.5 for chromatograms of individual runs with corresponding peak letters for each individual). Retention times (minutes) are displayed numerically on or above the corresponding peaks. Capital letters (A-I) indicate consistent and obvious peak duplications, whereas a lowercase "x" indicates other peaks that were not as consistent or easily classified. In the case of multiple peaks sharing the same letter (i.e. D1-D3); this refers to the number of peaks within the broader range of retention times that were present in an individual run. This was done (in the case of D, E and F) because there was a range of peaks that were highly variable in number (across individuals), but in which the whole range of peaks itself was easily identified. For example, there were 3 individuals that had 1 peak, 1 individual that had 2 peaks, and 4 individuals that had 3 peaks in the "D area" of elution patterns. There is no graphical representation in this figure of absorbance units of each peak (the relative volumes of the molecules at any given elution/retention time).

Discussion

The preliminary HPLC analysis of *P. cinereus* Mental Gland from the Virginia population revealed putative PRF and PMF components. That is, there are dominant peaks in the expected ranges for each of the known pheromone proteins (Figure 9). It should be noted that these molecules were not verified to be PRF, but are at this point considered "putative" (depicted as ~PRF/PMF) because of well-conserved similarities in their retention times to PRF and PMF from *P. shermani*,* which have been positively verified through proteomic (Feldhoff, unpublished data) and genetic analysis (Rollman et al., 1999; Palmer et al., 2007a; Houck et al., 2007a). The peaks in these regions not only demonstrate similar retention times to the pheromone proteins of *P. shermani*, but the absorbance values (and thus relative proportions) of these peaks are also similar to what would be expected.

There seem to be more peaks present in the elution profile of *P. cinereus* (compared to *P. shermani*), and one of these (RT=43.6) is the most dominant single peak (in this particular pooled sample). There is also a second, slightly less-dominant peak eluting shortly afterward (RT=46.6). Any guess as to the identities of these molecules would be mere speculation; however, there are a few potential candidates to consider. For one, the retention times of these unknown molecules are relatively close to that of the (non-PRF) protein "C3" of *P. shermani* (see Section I.A.8; also Figure 29). C3 in *P. shermani* elutes reliably at ~41 minutes, but it is possible that structural variation between species could account for slightly different retention times. There is currently very little information regarding the structure or

* A characteristic chromatogram of *P. shermani* MG extract can be seen in Section II.D (Figure 29), to which the reader is referred for a comparison to that of *P. cinereus* (Figure 9).

function of C3 (Feldhoff, pers. comm.), including whether or not it would be highly-conserved across species, especially those that diverged long enough ago (~27 MYA)* that they differ in pheromone delivery methods (scratching vs. olfactory).

A second consideration for this additional dominant peak is the ancestral pheromone component SPF (see Section I.A.8). SPF is thought to be undergoing gradual "evolutionary replacement" by PRF throughout the Plethodontids (Palmer et al., 2007b). This protein is expressed at very low levels in *P. shermani*, but is expressed strongly in more-removed Plethodontids, such as *Desmognathus, Aneides,* and *Eurycea* (Palmer et al., 2007b; Fontana et al., 2007; Kiemnec et al., 2009). Experimentally-applied SPF has been shown to increase female receptivity (decreasing courtship time) in *Desmognathus ocoee* (Houck et al., 2007b), much in the same way that PRF has been shown for *P. shermani* (Houck et al., 1998; Rollman et al., 1999). PRF is known to be under rapid positive selection (replacing SPF) in the eastern *Plethodon spp.* (Palmer et al., 2007b) and should be in higher proportion than SPF. Indeed, when combining the sum of the integrated area under all of the ~PRF isoform peaks (in Figure 9), the total ~PRF present is more abundant than the unknown peak. Most of the dominant peaks are accounted for in their position (with peaks from ~30-40 corresponding to the PMF region, and ~53-56 corresponding to PRF), and thus the remaining major components are the peak at ~43 minutes and another smaller one at ~46 minutes. SPF is the only known pheromone component remaining that is expected to be present in *P. cinereus*, and it could very well be one of these two unknown dominant peaks.

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* For these phylogenetic relationships see: Palmer et al., 2005; 2007b; Watts et al., 2004.

While the *P. cinereus* sample from the VA population exhibited an elution profile that was quite similar to what would be expected (based on that of *P. shermani*; Figure 29), the samples from the NH population were highly variable and poorly-resolved (see Appendix A.2; A.3). The histograms compiled from all NH-MG chromatograms (Figures 10 and 11) are crude attempts to illustrate the degree of consistency across all individual male samples. There were several peaks that reliably appeared, but it was not always the same peaks. In other words, many individuals were missing peaks of the total that appeared across all runs, and they were not always the same peaks missing. Even when the same peaks were present, the amplitudes/proportions of these peaks were highly variable. There were also a large number of peaks in the NH sample eluting less than 30 minutes, none of which were present in the VA sample (Figure 9). Of the eluents occurring after 30 minutes, there was little resemblance to the sample from VA. Lastly, the overall amplitude scale (AU) of the NH samples was very low, implying a low amount of total protein yielded in the extracts. Overall, the MG extracts from NH and VA appear to be drastically different; since the latter sample does in fact closely resemble the elution profile of *P. shermani*, the NH sample appears to be the outlier.

There are several factors (both biological and methodological) that could begin to explain the differences between *P. cinereus* samples. For one, the samples from NH were obtained in November, and the animals had been in laboratory conditions for many months. This could certainly have played a large factor. Pool and Dent (1977) found that secretory glands in the integument of *Notopthalmus viridescens* regressed to the non-breeding (non-hypertrophied) state after multiple

weeks in the laboratory. Assuming this remains true for Plethodontids, it could certainly be an important factor for the Mental Gland (which is hypertrophied seasonally; Sever, 1976b). Atrophied glands due to a long duration in laboratory conditions could also explain the low yield of total protein from the samples. It could be concluded that this factor alone was responsible for the observed differences between NH and VA samples; however, samples obtained the following spring (during the breeding season) from NH males (housed in the laboratory for only a few days before gland removal)* still did not completely resemble the VA sample (although it certainly looks more similar). Even so, the analysis of NH samples from different times of the year (after different laboratory-housing durations) revealed differing expression patterns (discussed in detail in Section II.C; see Figure 25).

Another factor that could be responsible for the differences between NH and VA samples is the geographic distance by which these populations are separated (more than 700 miles). There is known to be surprising differences in pheromone composition between different populations of *P. jordani*, even over drastically smaller distances than that separating the two populations of *P. cinereus* sampled. Rollman et al. (2000) tested the geographic variation of MG extracts from populations of *P. jordani* (within a 100 mile radius) and found significant differences in the relative proportions and the presence/absence of various glandular components between populations. This is extremely important when considering that at the time these populations were sampled, they were all considered sub-populations of one species, one of which has since been separated and renamed *P.*

* See RP-HPLC Analysis of Field-Quality males from Section II.C (Appendix A.7).

shermani (Highton and Peabody, 2000). This "species" has a drastically smaller geographic range than *P. cinereus* (Petranka, 1998), and yet even its sub-populations are highly different in the composition of their Mental Gland components.

There can be no doubt that the two populations of *P. cinereus* sampled have been isolated from each other for a long time, especially considering the small home-ranges (10-20 m^2; Kleeberger and Werner, 1982) and limited dispersal of these animals (typically less than 100 m; Marsh et al., 2007). There has also been a great deal of confusion in the taxonomy of *P. cinereus* in the past, with many authors disagreeing on the number of sub-species that should be identified (Highton, 1999).

An excellent example (analogous to the situation with *P. jordani/shermani*) is that of *P. serratus*: this species was for a long time considered conspecific to *P. cinereus*. It wasn't until phylogenetic studies investigating the geographic variation in protein patterns that the two "species" were uniquely distinguished (Highton and Webster, 1976). These researchers observed that the number of diagnostic morphological characters within this species complex were so minute that "the possibility of undescribed cryptic species must be considered seriously." Interestingly, the ranges currently ascribed to *P. serratus* are geographically isolated by substantial distances (Petranka, 1998), and the issue of species boundaries and classification is still one of surprising ambiguity. It could very well be that pheromone variation is one of the most important proximate mechanisms of speciation within the Urodela, with pheromone molecules acting as a premating isolating mechanism (Dawley, 1986b).

Recent research by Highton (1999) continued in this vein, proposing even more species distinctions. One of these newly-proposed species was *P. virginia*, existing in the Valley-and-Ridge geographic province of VA (which is the region from which this experimental sample was obtained). Essentially, there is a great deal of geographic protein variation that can take place intra- and interspecifically. Additionally, there is a great deal of ambiguity as to the exact species boundaries within the *P. cinereus* complex. Both of these factors could potentially indicate that the pheromonal composition of the *P. cinereus* from NH could be drastically different from that of the southern "*P. cinereus*." Even so, the VA sample would still be expected to be more similar to the northern *P. cinereus* sample than it was to *P. shermani*, even if it is from a different subspecies within the *P. cinereus* complex; however, that was not the case. Needless to say, this intriguing situation requires further research (and will be addressed further in Section II.D).

The HPLC analysis of the PCG of *P. cinereus* from NH (see Appendix A.4; A.5) was the first of these analyses done, and as such there are no other samples from different species or conspecific populations with which to compare. The results of this analysis revealed a variety of well-conserved peaks across all individuals, mainly between ~39-49 minute retention times. Surprisingly, the variation across all PCG samples seemed to be more highly-conserved than the variability between MG samples (Appendix A.3 vs. A.5). More detailed information about the exact components observed will be presented in the following section (II.B.2; after SDS-PAGE analysis) and in Section II.C (after additional HPLC analysis of the PCG from Field-Quality males).

II.B.2. SDS-PAGE ANALYSIS OF PHEROMONE COMPONENTS (MG + PCG)

Methods

SDS-PAGE Analysis was conducted on MG and PCG extracts. Technically these samples were from the "Field Quality" animals used in Section II.C; see page 107 for animal collection information. The surgical and extraction protocols were identical to those used in the above RP-HPLC analysis (Section II.B.1). A bicinchoninic acid (BCA) protein assay was done on each sample to quantify the concentration of protein present (Smith et al., 1985). Protein concentrations were then used to load a predetermined mass of proteinaceous sample into polyacrylamide gels for electrophoresis. This was done by taking the appropriate volume of a given sample (depending on the density of protein), drying it down (SpeedVac), and resuspending it in 15μL of dH_2O. Samples (see below for mass loaded in each case) were run on 15% Tris-Tricine gels at 100V for 75 minutes. All gels (unless otherwise noted) were run in duplicate: in the presence and absence of reducing agent (+/-). All gels were stained with Coomassie blue.

The first sample run was a pooled MG extract from *P. shermani*, which had been collected by Dr. Feldhoff and Dr. Lynne Houck in August, 2008. This was done to serve as a baseline for the MG components that have been previously investigated at the biochemical level, and as a comparison for how similar the components of the MG extracts of *P. cinereus* are to the more tested system. The protein mass loaded into adjacent lanes were: 5μg, 10μg, 15μg and 20μg. A second gel consisted of one lane of MG extract (15μg; limited by low protein concentrations) and two lanes of PCG extract (10 and 20μg).

A third gel was utilized in conjunction with RP-HPLC analysis to determine the protein sizes corresponding to two major peaks within the PCG sample. This was done by manually collecting the sample as it eluted from the column. Since RP-HPLC separates molecules based on hydrophobicity, using in conjunction with SDS-PAGE enables the identification of how many different proteins are present and what sizes they are. In this case, individual peaks were run to identify the size of the proteins that make up the majority of the PCG sample (E and F fractions; see Appendix A.6).

Lastly, two gels were run containing individual PCG extracts (as opposed to pooled samples). This provides the benefit of added resolution between components, as molecules in large proportion in an individual sample can become overrepresented in a pooled sample of multiple individuals. This also allowed for analysis of the degree of individual variation in PCG secretions. A standard mass was loaded for each individual (20μg), so the changes in staining density of specific bands represent the differences in relative ratios of pheromone components. One gel contained the 7 largest animals of the Field-Quality sample, while the second gel contained the 7 smallest animals. The main goal was to observe variations between individuals in general, but in this way it allowed for observation of any potential changing component ratios between the two polarities of the Field Quality size-distribution.

Results

Upon analyzing the *P. shermani* MG extract (Figure 12), the results yielded separation of proteins consistent with past research in this area (Rollman et al., 1999; Feldhoff et al., 1999). The two major proteinaceous components can easily be seen: PRF (22 kDa) and PMF (7 kDa).

The MG extract for *P. cinereus* does not demonstrate obvious bands for either of these proteins (Figure 13; lane 2), though the resolution of separate bands is poor. The major proteins of the MG extract appear at ~66 kDa, and at ~10 kDa.* The ~66 kDa band is present in the *P. shermani* MG sample, and in both cases it appears to actually consist of two distinct bands (this is easier to see in *P. shermani*; Figure 12). There also appear to be numerous small bands present with mass $\geq$ 20 kDa, and again with mass $\leq$ 14.7 kDa. The PCG extract has numerous large bands, and seems to have more proteins present, and in more even proportions (Figure 13; lane 3). There is once again two bands present at ~66 kDa (as in the MG of both species). Other dominant bands in the PCG extract occur: ~36-45 kDa, ~20-25 kDa, ~16 kDa, ~10 kDa and ~7 kDa. There is some overlap between the PCG and MG of *P. cinereus* (Figure 13), but there are also similar bands between the PCG of *P. cinereus* and the MG of *P. shermani* (Figure 13, lane 3; Figure 12).

In comparing the PCG extract in the presence (+) and absence (-) of a reducing agent, the only difference observed is that when there is no reducing agent, a much darker band can be seen at ~28 kDa (see 20μg PCG; Figure 14 vs. 15). In the

*All molecular weights to be stated under *P. cinereus* SDS-PAGE analysis are estimations based on the size-ladder standard, and are not accurate in-tact masses until verified by mass spectrometry (as has been done with PRF and PMF of *P. shermani*).

presence of reducing agent, disulfide bonds (S-S) are broken, which unfolds cross linkages and can split larger proteins into their subsets. In the (+) gel, there is mostly the band at ~14 kDa, with only a very faint band at ~28 kDa. In the (-) gel, there is a great deal more protein at ~28 kDa. Thus, it seems that when reduced (S-S bonds broken), the larger protein breaks down into two smaller pieces. These two pieces seem to be half of the size of the larger, and therefore may represent a dimerization.

After collecting the two major components of the PCG from the RP-HPLC (which seem to correspond to fractions E and F of Section II.B.1.)* and running them on a gel, it concludes that the E-fraction has a single band at ~10-14 kDa, while the F-fraction consists of two bands: ~66 kDa and ~7 kDa (Figure 14). When running these same samples without reducing agent (Figure 15), it indicates that the protein which seems to partially exist as a dimer (putative; see above paragraph) is present in the E-fraction of the RP-HPLC chromatograms.

Lastly, when separating the extracts of individual male PCGs (Figure 16), there are a number of bands that vary in their relative abundance between different males: two bands between 36-45 kDa, ~24 kDa, one band > 14 kDa and one band between 7-14 kDa. The band slightly larger than 14 kDa seems to be particularly variable, espcially between larger and smaller males, though this is merely a casual observation at this time.

* See Appendix A.6 for the chromatogram from which these fractions were collected. The times for the fractions are not identical (as these were from different animals, and run at different times), but through comparing relative elution times of the characteristic peaks, it seems that these correspond to the E- and F-fractions of Section II.B.1.

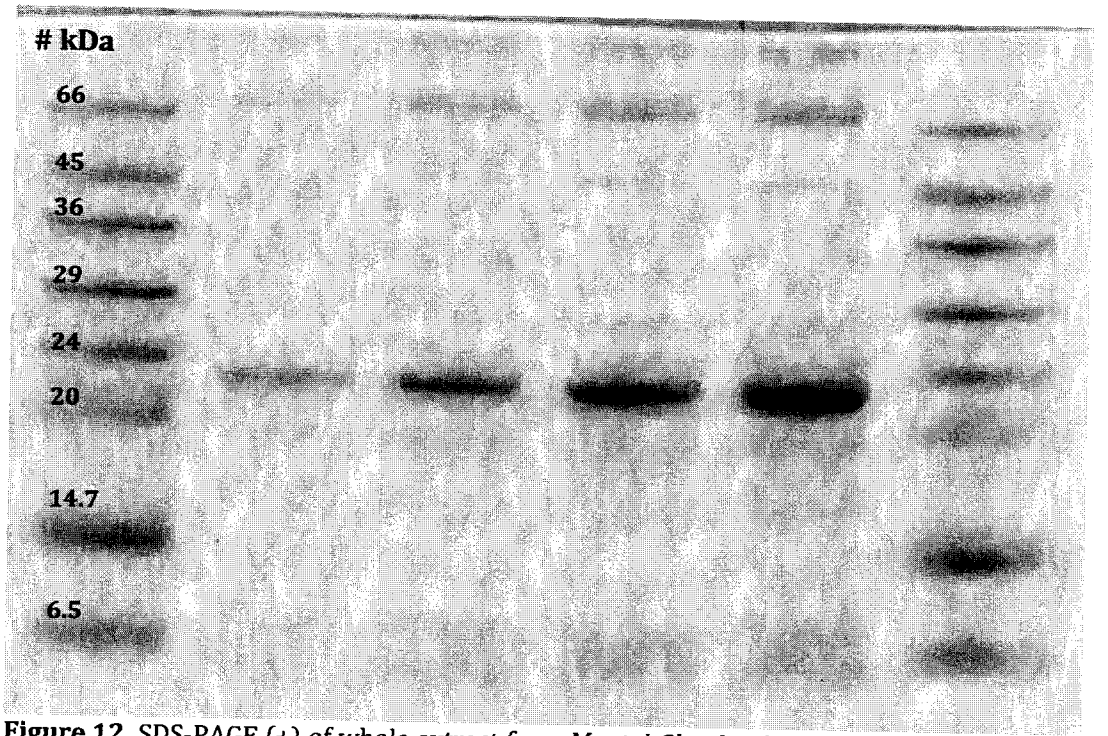


Figure 12. SDS-PAGE (+) of whole extract from Mental Glands of *P. shermani*. Lanes 2-5 are increasing concentrations of protein loaded into the wells (5, 10, 15 and 20μg respectively). Lanes 1 and 6 are size-ladder standards of known proteins to be used as a comparative measure to estimate the size of unknown proteins in the samples. The size (kDa) of the bands in the standard are labelled on the left ladder. The large, predominant band in the MG sample is PRF (all isoforms), which is 22 kDa in size. PMF is also visible as a wide, poorly-resolved band in the 7 kDa area. The poor resolution of PMF relates to the drastic structural variability of this pheromone component. These components make up 85% of the mixture.

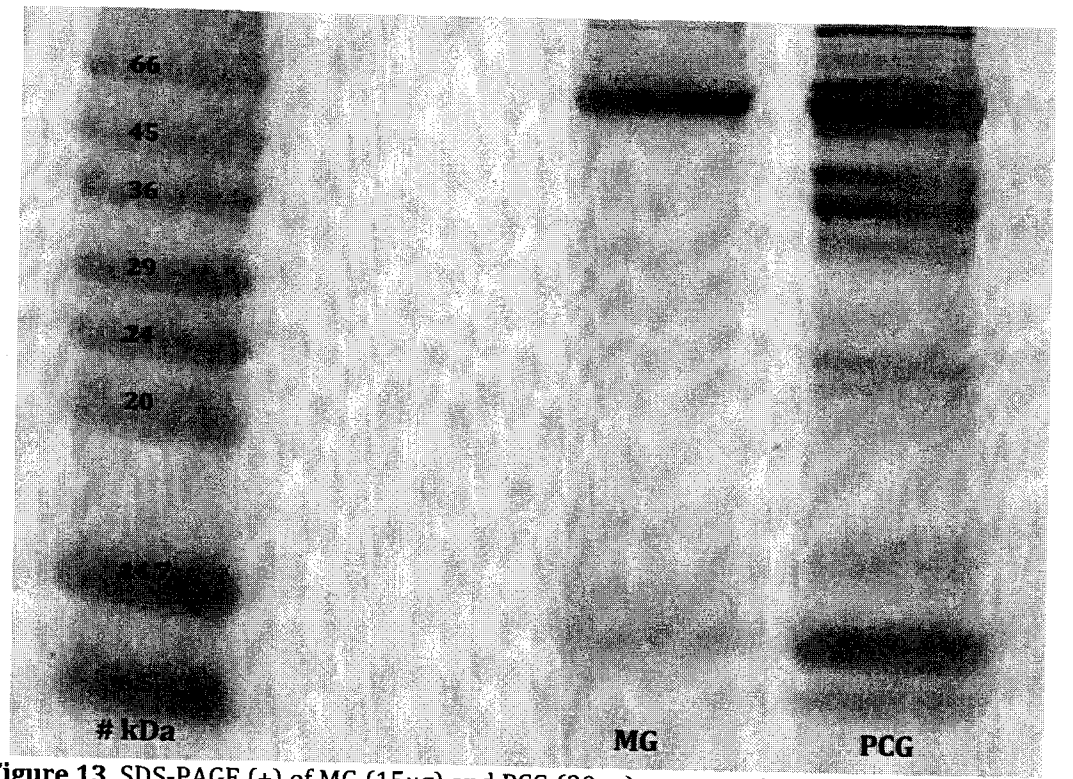


Figure 13. SDS-PAGE (+) of MG (15μg) and PCG (20μg) extracts of *P. cinereus*. Present in the MG are dominant bands at ~66 kDa (actually be two bands; see PCG) and ~14 kDa. There are numerous faint bands present in the range between these components, and the disparity between major and minor components is partly heightened by the pooling of the samples from all individuals. In the PCG, there are numerous proteins of differing sizes, all in more equal proportions of volume. There appears to be some overlap between the two glands in the types of proteins present, though there are also a number of differences.

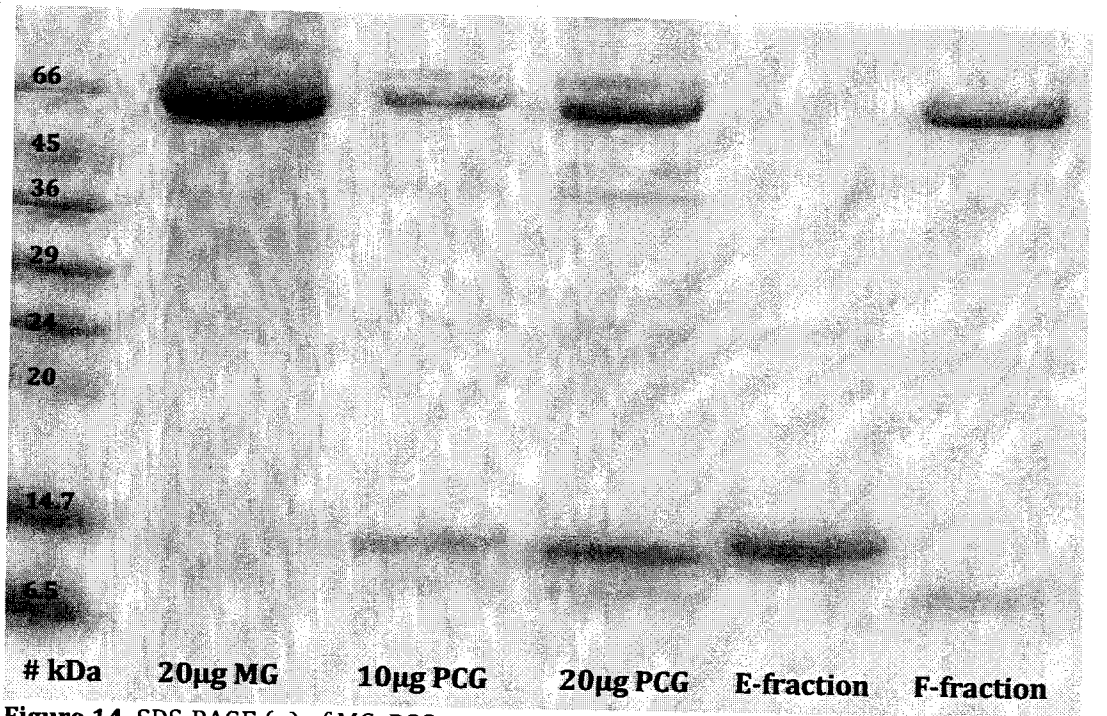
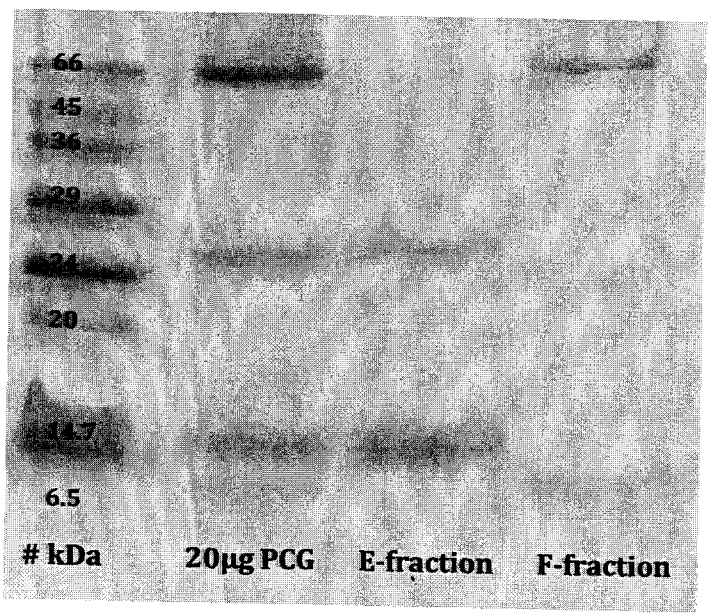


Figure 14. SDS-PAGE (+) of MG, PCG extracts, as well as the two major component fractions from RP-HPLC analysis. The fraction that appears to coincide with the E-fraction (of Section II.B.1) is a single protein of ~14kDa, while the F-fraction consists of two proteins of sizes ~66 kDa and ~7 kDa.

Figure 15. SDS-PAGE (-) of PCG whole extract next to the E- and F-fractions collected from the RP-HPLC column. These are the same samples as lanes 4, 5 and 6 of Figure 14, but without reducing agent. This indicates that the middle band of the PCG extract (~28 kDa) can be separated when reduced (S-S bonds broken), and implies that this molecule may exist as a both a dimer and a singular form. This band is now known to elute in the E-fraction from the RP-HPLC.



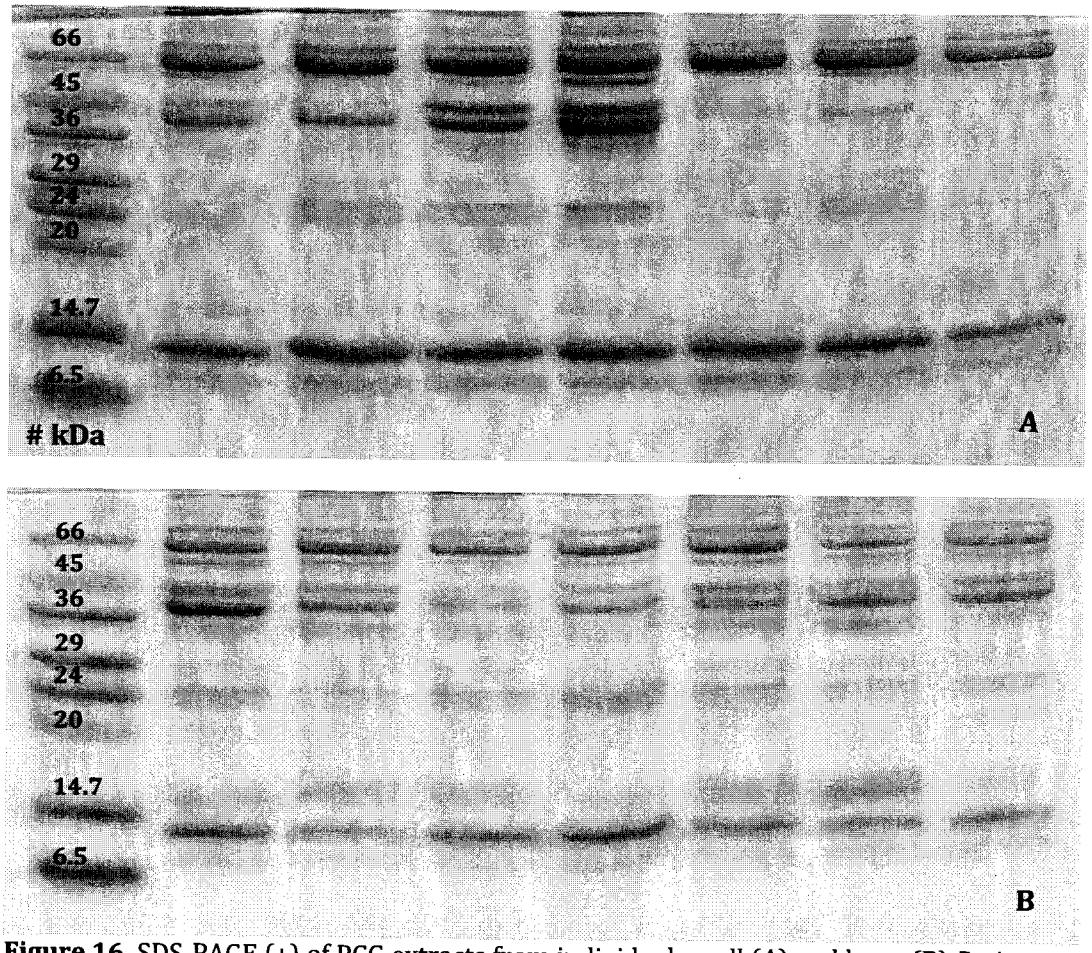


Figure 16. SDS-PAGE (+) of PCG extracts from individual small (A) and large (B) *P. cinereus*. All lanes contained 20μg of protein, so the changes in staining intensity indicate changes of relative component ratios. There are numerous bands that appear to vary drastically between individuals, specifically: two bands between 36-45 kDa, ~24 kDa, and a band between 7-14 kDa. One band in particular (greater than 14 kDa) appears to be more prevalent in larger males, though this is merely casual observation at this point in time.

Discussion

The SDS-PAGE analysis of the *P. shermani* MG (Figure 12) provided a reliable baseline with which to compare the MG extracts of *P. cinereus*, since the major components of the mixture have been identified in the red-legged salamander. PRF is demonstrated as a large, dark band with a size of 22 kDa. PMF can also be easily seen surrounding the ~7 kDa area of the gel. While PRF appears to be the most dominant component on the gel (and for a single band it is), PMF is actually known to compose the majority of the mixture. Again, 85% of the total pheromone composition of *P. shermani* consists of PMF and PRF, in a 2:1 ratio respectively (Houck et al., 2007a; Feldhoff et al., 1999; Fontana et al., 2007). Thus, the total of PMF is twice as abundant as the total of all PRF protein. The *P. shermani* MG gel appears misleading in this regard because the totality of PMF is spread out over a wider band due to an immense degree of structural variability (described as "hypervariable"; Houck et al., 2007a). The third identified proteinaceous component can also be observed (particularly in the higher concentrations): a band representing C3 can be seen at 18 kDa. Additional components are distributed throughout the remainder of the gel, and the most abundant of these is a dual band at ~66 kDa.

When comparing these results to the MG analysis of *P. cinereus* (Figure 13), there are a few bands that overlap. The largest of these is the dual band occurring at ~66 kDa. These bands can also be observed in the PCG of *P. cinereus* (Figure 13). A second component of the *P. cinereus* MG can be observed at ~10 kDa, and could potentially be considered within the range of PMF (originally described as "Pj-10"

because it appeared to be roughly 10 kDa large; e.g. Rollman et al., 1999; Feldhoff et al., 1999; Rollman et al., 2000). Additionally, the *P. cinereus* band occurring in this range is also a wide, diffuse band (as is the PMF of *P. shermani*); this indicates a similarly wide degree of structural variability that would be expected of PMF. The main problem with drawing any conclusions about the MG of *P. cinereus* is that the resolution between bands was extremely poor. The main obstacle encountered with the *P. cinereus* MG is that the total protein yield was very low, even in the "Field-Quality" animals of Section II.C (from which these samples were taken). These animals were only in laboratory housing conditions for 5-10 days each, which is not enough time for the secretory glands to regress to the non-breeding state (as per Pool and Dent, 1977), which was thought to be the cause of the low protein yield of Section II.A.1. These animals were also obtained during the peak of the breeding season, so it would be expected that their Mental Glands would be in optimal form for courtship. Even so, the protein yield was very low, and was therefore a limiting factor in running the gels. This was mainly a problem because there was not enough protein obtained from a single animal to acquire any noticeable staining. Thus, all MG samples had to be run as a pool; in a pooled sample, however, the resolution between peaks is diminished: proteins in greater abundance become overrepresented, while the less-abundant proteins become overshadowed.

Unfortunately, this limitation hindered the ability to clearly establish definite boundaries between bands in the gel (although the MG separation of Figure 14 is better than that of Figure 13). All the same, it is still possible to see some similarities between both the MG analysis of *P. shermani* and the PCG analysis of *P. cinereus*.

Regarding the former, it seems that there could in theory be PMF observed, although it did not show up overwhelmingly in the RP-HPLC analysis of the NH sample* (Appendix A.2) where it would be expected (RT=30-40 min.). This does not necessarily rule out the presence of PMF in the *P. cinereus* sample from NH, because the hydrophobicity of PMF could be different between species and conspecific populations (due to structural differences), and thus their elution times on the RP-HPLC column would differ. The presence of PMF is expected in *P. cinereus* based upon genetic analysis (Palmer et al., 2009; Kiemnec et al., 2009).

PRF would also be expected to be present in *P. cinereus* (Watts et al., 2004; Palmer et al., 2005; 2007b; Kiemnec et al., 2009). Its presence in the SDS-PAGE analysis (Figure 13/14) is not definitive, yet it seems that there are faint bands present in the vicinity of 20-24 kDa. Like PMF, the initial RP-HPLC analysis of NH *P. cinereus* did not reveal peaks in the vicinity of PRF (although it did for the VA sample). However, these gels were run with samples from the Field-Quality males of Section II.C, and the RP-HPLC analysis of these animals did demonstrate a shift in expression patterns (from the previous NH samples) that did display a peak in the vicinity of PRF (see Figure 25). There is therefore an indication that PRF may be present in the MG of *P. cinereus* from NH. Not only should PRF be present in the region from 20-24 kDa, but SPF should also be present. It is again known that SPF is present in the *P. cinereus* MG due to genetic analysis (Watts et al., 2004; Palmer et al., 2007b; Kiemnec et al., 2009), and should therefore appear at 23 kDa.

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* While dominant peaks in the PMF region (of RP-HPLC) did not show up in the NH sample of *P. cinereus* (Appendix A.2), from which these gels were run, it did in fact show up in the VA sample where expected. Thus, it may be that geographic variation is still at play (see Discussion of Section II.B.1).

The reason that the bands in the PRF and SPF region do not appear clearly in the SDS-PAGE analysis may be due to the huge proportion of the dual band occurring around 66 kDa. The identity of these molecules is unknown, although it is present in all of the samples tested (*P. cinereus* MG/PCG; *P. shermani* MG). In a situation in which a set mass of protein is loaded, a single band which is extremely abundant will leave only a small fraction of the remaining mass to be occupied by the entirety of the remaining proteins, which are therefore very poorly represented. In this way, the proteins with sizes less than ~60 kDa could not be resolved to the degree necessary, but it is at least clear that there are several faint bands observed throughout the size separation. Unfortunately the low protein yield of the *P. cinereus* MG left no additional protein for further analysis (between the SDS-PAGE and RP-HPLC analyses completed); the need for these analyses did not allow for the use of a tremendous proportion of the total volume of MG sample which would be required in order to obtain the large amount of mass needed to clearly demonstrate the underrepresented components. Future research will amend this, and more clearly identify the exact protein number and sizes in the *P. cinereus* Mental Gland.

The SDS-PAGE analysis of the pooled PCG sample (from NH *P. cinereus*) demonstrated a much greater degree of resolution between bands. In this situation, there was a great deal more protein with which to work (an entire order of magnitude more than the MG; see Section II.C, Table 1), and the ability to run PCG samples from individual males was possible, which allowed for the greatest resolution of bands (Figure 16; discussed below). Even in the pooled PCG sample (Figure 13/14/15) the relative proportions of multiple bands were more similar to

each other, and thus one extremely abundant band did not overshadow the other components. It was therefore clear that there were a great number of proteinaceous secretions from the ventral caudal integument, the Postcloacal Gland.

Of these, one of the most dominant components was still the dual band occurring at ~66 kDa. There were also three abundant bands between 30-45 kDa, two/three bands between 20-24 kDa, and three remaining bands occurring around ~15, 10 and 7 kDa (the resolution of components is easiest to observe in individual PCG samples; Figure 16). While it is not thought that courtship pheromones are present elsewhere than the MG (in *P. shermani*; Palmer et al., 2007a; Fontana et al., 2007), it does appear that there are two bands occurring at ~22 and ~23 kDa (the sizes of PRF and SPF respectively) in the PCG of *P. cinereus*. There is also a band that seems to occur at ~7 kDa (the size of PMF).

Palmer et al. (2007a) did in fact identify small amounts of PMF mRNA in the caudal integument of *P. shermani* via RT-PCR, though Fontana et al. (2007) did not find significant amounts through ISH labeling. These differences may be due to the fact that RT-PCR is a more sensitive methodology. Palmer et al. (2007a) also identified low levels of PMF mRNA in the liver, kidneys and intestines in both sexes. While Fontana et al. (2007) concluded that there was no significant labeling of PRF, SPF or PMF anywhere in the body other than the MG, their results were somewhat inconsistent. The presence of SPF varied greatly between and within individuals: some were completely negative, while others labeled strongly. SPF was also shown to be present in the epidermis of the MG (and other regions of the body), while no PRF or PMF was found in the epidermis (but rather in dermal mental glands only).

Multiple (but not all) individuals also had some other various labeling of PMF and SPF (in "flask cells"). However, the probes utilized in the ISH assay by Fontana et al. (2007) used the coding region of the mRNA; in most cases this region is normally the most conserved, but in the unique case of PMF, the coding region is actually highly variable compared to the untranslated region (UTR). The probe was also geared towards a single isoform. Thus, this methodology could have misrepresented the degree of PMF expression in non-MG tissues (D. Wilburn, pers. comm.).

The inconsistencies of these findings in *P. shermani* (especially considering the conclusions of Palmer et al., 2007a) can therefore hardly rule out the possibility of PMF expression outside of the Mental Gland in *P. cinereus*. It also seems likely that SPF could be more widely-distributed than the MG (and it is thought to be more prevalent in non-*P. shermani* Plethodontids; Watts et al., 2004; Palmer et al., 2007b; Kiemnec et al., 2009). Thus, further analysis of the exact glandular secretions of the PCG should be carried out to confirm or disprove the identity of various components.

The secretions of the PCG were also explored regarding the relationship between the SDS-PAGE and RP-HPLC analyses. As previously addressed, the samples utilized for this SDS-PAGE analysis came from the Field-Quality males of Section II.C, which were also utilized for further RP-HPLC analysis.* For this HPLC analysis (drawing on the conclusions of Section II.B.1), the PCG samples were passed through a filter which maintained only proteins larger than 3 kDa. This allowed other small, non-proteinaceous molecules (which are abundant in the PCG; Hecker et al., 2003) to be removed from the HPLC analysis, resulting in only the

* The details of this RP-HPLC analysis can be seen on page 124 (also see Appendix A.10).

demonstration of protein components (> 3 kDa). This resulted in two dominant peaks (see Figure 28), which were collected for SDS-PAGE analysis (Figure 14/15).

In this way, it is possible to identify which bands in the gel (protein sizes) correspond to which peaks in the RP-HPLC chromatogram (hydrophobicity). It was revealed that the major peak (~43 minutes; corresponds to the E-fraction of Section II.A.1) consisted of a single protein with a molecular weight of ~10-14 kDa. The second peak (~46 minutes; corresponds to the F-fraction of Section II.A.1) was shown to consist of two different proteins. These proteins are of different size and sequence, but share similar hydrophobicity and thus elute at the same time from the RP-HPLC column. These proteins in the F-fraction consist of the largest (~66 kDa), as well as the smallest (~7 kDa) proteins of the total PCG mixture. It is at this point clear which bands elute at which time on the RP-HPLC (of those discussed). There are, however, more bands present in both analysis than were correlated between the two analyses.

The PCG extracts were also the only gels that demonstrated any differences in the presence (Figure 14) and absence (Figure 15) of reducing agent (+/-). The only protein of the PCG mixture in which a difference was noted was the band at ~10-14 kDa. In the presence of reducing agent (+) it consisted of a single band, while in the absence of reducing agent (-) it consisted of two bands: one at ~10-14 kDa and another at ~24 kDa. In the (-) gel, the extra band appears to be twice as large as the original band. This indicates that this protein could potentially be forming a self-dimer. When there is a reducing agent to break disulfide (S-S) bonds, there is no trace of the doubly-sized protein; however, it does appear when there is

no reducing agent to break disulfide bonds. Thus, it seems that the disulfide bonds are responsible for the large size of this protein, and since it is twice the size, it seems highly likely that it is a self-dimer: two of the smaller proteins bonded together. Whether this is an experimental artifact (possibly from the process of "drying down" the proteins) or a biological function of this protein is unknown.

There is only one other study that has analyzed the secretions of the caudal integument with this method to my knowledge (using *P. shermani*). In this research, Largen and Woodley (2008) demonstrated (among other things) the difference between adhesive skin secretions (which come largely from the dorsal and lateral surfaces of the tail) vs. the secretions from the ventral portion of the tail (responsible for scent-marking). The issue of adhesive secretions could still technically be present in the current analysis, though it is likely a minimal one because of the location of skin removed and the specialization of this glandular area. Also, all sections of skin were rinsed initially in PBS prior to being placed in the extraction solution. Still, it could be that some of the proteins demonstrated are of non-communicative function, some of which could be geared towards predator-defense. However, anti-predator secretions were shown to have very little overlap with the ventral tail secretions (Largen and Woodley, 2008), and are therefore not likely to be a major component of the PCG analysis of the current experiment.

Lastly, the analysis of PCG extracts from individual males revealed not only a greater resolving power, but also a tremendous amount of variability in the pheromone profiles of individual males (Figure 16). This is of great importance to the underlying hypothesis of this research: individual variation must be expressed

within the composition of the pheromone mixtures in order to encode any information that varies between senders. Since it is known that Postcloacal secretions confer a great deal of sender-specific information to the receiver (see Section I.A.4), it is likely that the high degree of protein variability demonstrated is playing a large role in facilitating the transfer of information. It should not be assumed, however, that protein variability is the only mechanism responsible, as other molecules such as carbohydrates and lipids are: (1) also known to be secreted by these glands (Hecker et al., 2003), and (2) capable of conveying variable information through subtler degrees of structural plasticity (Alberts, 1990).

It should also be remembered that the different bands of an SDS-PAGE gel are completely different proteins. If different proteins have different functions, it can certainly be seen how changes in the relative proportions between these various proteins could facilitate different effects on the receiving individual. Inversely, there is also typically a great deal of structural variability within a specific protein (i.e. PMF; Palmer et al., 2007a), and this could very well be a second means of conveying different gradations of a variable series of information. The scale of SDS-PAGE simply does not allow for such fine-tune separation (although the width of the band can indicate this to some degree, e.g. PMF; Figure 12). The use of RP-HPLC does allow for separation of smaller-scale structural variations, and the combination of these methods is therefore highly useful: electrophoresis allows for accurate assessment of the relative proportions between proteins, while HPLC can allow for the separation of different isoforms of the same protein. Of course, the challenge in the latter case is the identification and verification of the exact isoforms, as well as

the fact that often multiple proteins can share close enough hydrophobicity that they elute at the same time and skew the information of the amount of a specific eluent (i.e. the F-fraction; Appendix A.6 vs. Figure 14/15).

In summary, additional research is needed to identify the exact mechanisms by which individual-specific information and gradations within a data-series can be conveyed through changing ratios of pheromone components. Overall, the data of this preliminary biochemical analysis has provided a great deal of insight into the nature of these pheromone mixtures: the number and sizes of proteins that are present in different tissues, how they compare to the previously-established model system (*P. shermani*), and how the signals differ between individuals. While there is still a great deal more to be learned, this experiment has provided an essential step towards important future experimentation.

II.C. DIFFERENTIAL DIET STUDY: BIOCHEMICAL AND BEHAVIORAL ANALYSIS

Objective

The goal of this experiment was to investigate how certain changes at the biochemical level could act as a mechanism for the behavioral function of conveying information about diet-quality that has been demonstrated in previous experimentation (Section II.B.3). Female Plethodontids have been shown to infer information about diet-quality over an incredibly short period of time with only the pheromones secreted via scent-marking behavior (Figure 5), but also more indirectly and over a longer period of time through the inference of size (Mathis, 1990; 1991; Marco et al., 1998; Verrell, 1995). Specifically, the first objective of the experiment was to test the hypothesis that diet-quality can be conveyed as a graded signal through territorial advertisements, with better fed males simply producing larger quantities of all scent marking pheromones. It was therefore predicted that males placed on High-Quality diets would have more protein present in their secretions than those placed on Low-Quality diets.

While it is clear that individuals are capable of inferring this information through pheromones, it remains to be seen what the role of the individual glands are in facilitating this function. Mental Glands are utilized for courtship only, while Postcloacal Glands are utilized for territorial advertisement through scent-marking (Simons et al., 1994; 1999). Since conspecifics are able to infer a great deal of information from these advertisements without any visual information from the senders (see Section I.A.4), it seems highly likely that the Postcloacal Gland may be responsible for conveying information about diet-quality. Similarly, having a higher

amount of energy to invest into the production of courtship pheromones could also be of great advantage to courting males, since more molecules would lead to increased sensory stimulation and a stronger physiological response from females, making them more likely to mate. It was therefore predicted that the High Quality males would also have more Mental Gland protein than Low-Quality males.

While the concentration of pheromone proteins between diet-groups will provide insight into whether or not the signal of diet-quality is graded, it does not answer the question of whether or not there are changes in relative pheromone ratios that could also convey different aspects of the total information. The degree of complexity within this pheromone system requires a tremendous amount of structural and communicative plasticity, and therefore multiple forms are likely to be at play. This is particularly relevant when considering that of the two known pheromone proteins, both have multiple structural variants (PRF - Rollman et al., 1999; PMF - Palmer et al., 2007a). At the same time, not all of the degrees of complexity within the signaling system are required to be information about changing environmental conditions. It is also known that there is an element of population variation in pheromone composition (Rollman et al., 2000), and through behavioral analysis it is clear that aspects such as species (Dawley, 1984), sex (Jaeger et al., 1986), reproductive status (Dantzer and Jaeger, 2007b), familiarity (i.e. individual recognition) and smaller scale spatial information (Madison, 1975; McGavin, 1978; Jaeger, 1981) all need to be represented through the pheromone variability.

Therefore, the second goal of this research was to identify how changes in environmental conditions (i.e. diet-quality) can modify the relative ratios of specific components of the total pheromone mixture. It was hypothesized that changes in environmental conditions can modulate the pheromone profiles of the source individuals, since a variety of environmental conditions are inferred solely based on pheromones. Observation of these potential changes will identify which specific molecules within the total mixture may be responsible* for conveying this specific aspect of environmental information (diet-quality).

The first hypothesis was tested through a BCA protein assay of Mental and Postcloacal protein from High- and Low-Quality males, while the second hypothesis was tested through RP-HPLC analysis in an attempt to investigate any changes in ratios of pheromone components associated with diet-quality.

Methods

Salamanders were collected in May, 2008 from Kingman Farm in Durham, NH (IACUC #080301) and were housed in the lab at 20°C on a 14:10 L:D cycle (IACUC #080501) in small plastic containers (15 x 15 x 5 cm) with moist paper towels as substrate and shelter. Animals were measured (TL, SVL; mm) and weighed (g). Two separate sample populations of males were identified for different biochemical and behavioral analyses (described below). One population consisted of individuals (n=22) of varying sizes. These males had no experimental manipulations, and were considered the "Field Quality" sample. The purpose of this sample was to

* Technically these data will be correlational, and by definition can't actually conclude causality. This is always important to keep in mind about correlational data, but even so this information will provide a tremendous initial leap in this area of study.

serve as a baseline for the normal range of biochemical variation found in the overall population. Thus, gland removal and pheromone extraction were commenced as quickly as possible after capture. The only manipulation undergone by these individuals was laboratory-condition housing for a short period of time (from 5-10 days in laboratory conditions*). Mental and Postcloacal Glands were surgically removed to be analyzed biochemically (see methods below). This would act as a comparative control for the biochemical changes to be observed after manipulation of diet-quality.

The second sample population consisted of males to be placed on different diets for a period of six weeks ("Diet-Quality" group). Diet-Quality males were size-matched (+/- 0 mm SVL; +/- 2 mm TL) to eliminate the variable of size prior to differential feeding. After size-matching, both males of the pair were placed onto different diets (High- and Low-Quality) in a manner that even subtle weight differences between males of the pair were evenly balanced across both treatments prior to feeding. High-Quality males were fed 0.1g of *Enchytraeus* white worms, while Low-Quality males were fed 12-14 *Drosophila* fruit flies. Feeding occurred on the same day once a week, and continued for six weeks. At this point, males were starved for one week to allow all food to pass through their gut, and avoid the possibility of fecal contamination of the body-wash water.

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* This was only limited by how quickly surgical procedures and biochemical extractions could take place. Because the objective was to obtain the glands from males as close to the normal field condition as possible, sexing was not done after two weeks of starvation (as in past experimentation). These males were therefore starved for two weeks after the gland removal surgeries, to verify the sex prior to biochemical analysis. At this point, greater experience had allowed for more accurate sexing without the need for a non-compulsory starvation period; all individuals were later verified to indeed be males.

On the eighth week (six weeks of feeding, one week starvation), novel gravid females (n=12) were tested for behavioral preference between the scents of High- and Low-Quality males. While the findings of Section II.A.3 indicate that females can infer this information through pheromones in a surprisingly short period of time, this behavioral analysis was to be used as a verification of that conclusion (and the longer-term findings of Section II.A.1). In addition, this serves as a confirmation that this behavioral function is actually happening within the same exact sample of animals on which biochemical analyses were to be completed. This avoids the reliance on any assumptions that the potential biochemical differences to be seen were related to the female detection of diet-quality.

On the day of testing, males were placed into 5mL of body-wash water in a small Petri dish (5 cm diameter x 1 cm) with holes drilled into the cover for ventilation. Males remained in the body-wash water for six hours, at which point they were removed and returned to their home container. The testing apparatus (identical to Section II.A.1) was an open plastic container (14 x 19 x 10 cm). The sides were covered with clean moist paper towels, with 8 cm of neutral area in the middle which had no paper towel covering it. At the beginning of a trial, the female was placed into the neutral center area under a habituation cup for five minutes to allow acclimation to the testing chamber. During this time, the 5mL body-wash water from the two males of a pair was placed onto either side of the chamber. The order of source-male pairs to be tested was randomized, as was the side of the container to receive each scent. After the five minute habituation period had passed, the habituation cup was lifted and timing commenced. Females were observed with

a continuous focal sampling method for 5 minutes, and were scored for Time-in-Proximity and the number of Nose Taps. Time-in-Proximity was again defined as having the head and both front limbs on the paper on a given side of the container. The number of Nose Taps was analyzed independently, but also when compensated for the amount of time on a given side (Investigatory Rate; NT/min). All data were analyzed with a two-tailed T-test assuming unequal variance.

After behavioral verification of female preference for the scent of High-Quality males, the Diet-Quality group also had their Mental and Postcloacal Glands surgically removed and their pheromones extracted. Surgeries for the Field-Quality males took place in early May, while for the Diet-Quality group it occurred in early July. Diet-quality males were also re-weighed prior to surgery to identify changes in weight associated with six weeks of differential feeding.

Surgical procedures and pheromone extraction methods were identical to those used in Section II.B. In brief, animals were anesthetized in 7% ether for ~10 minutes, at which point Mental and Postcloacal glands were surgically removed. The glands were placed into 200μL of AchCl extraction solution to initiate the release of granular gland secretions. Extractions were then centrifuged (14,000x) for three cycles of ten minutes, continuing to remove the supernatant and place it into a clean vial. After centrifugation, extracts were frozen at -20°C. Solutions were then brought to the lab of Dr. Richard Feldhoff (University of Louisville, KY). MG and PCG extracts of all animals were syringe-filtered (to remove large polymers) and passed through a 3kDa Centrifugal Ultrafiltration (only proteins >3kDa). Samples were tested for protein with a BCA assay and analyzed via RP-HPLC (see Section II.B.1 for methods).

Results

Effects of Diet: Weight Gain/Loss

Salamanders raised on different diets had statistically significant changes to their weight (Figure 17). Prior to differential feeding, there was no difference between High- and Low-Quality diets (two-tailed T-test; df=21; t=0.6; p=0.52). After six weeks of being fed different diets, there was a statistically significant difference between the weights of High- and Low-Quality males (df=22; t=6.9; p<0.00001). When comparing the weights of individuals within-group, there are significant differences in weight before and after feeding. High-Quality males weighed significantly more after six-weeks of feeding, gaining an average of 0.165g (df=11; t=4.5; p<0.001). Low-Quality males weighed significantly less after six-weeks of feeding, losing an average of 0.178g (df=11; t=8.4; p<0.00001).

Female Preference Verification

As predicted, females were able to infer this information through chemical signaling, as determined by their increased investigatory behavior for the scent of High-Quality males (Figure 19). Females did not spend a significantly more Time-in-Proximity to the scent of High-Quality males (df=21; t=1.6; p=0.13). Interestingly, while this seems in contradiction to the hypothesis, it appears to be due to unexpected differences at the biochemical level that only strengthen the hypothesis further (see Discussion). Females did, however, exhibit a significantly greater number of Nose Taps to the High-Quality scents (df=13; t=3.2; p=0.007), and also investigated the scents at a significantly faster rate (NT/min; df=17; t=2.3; p=0.03).

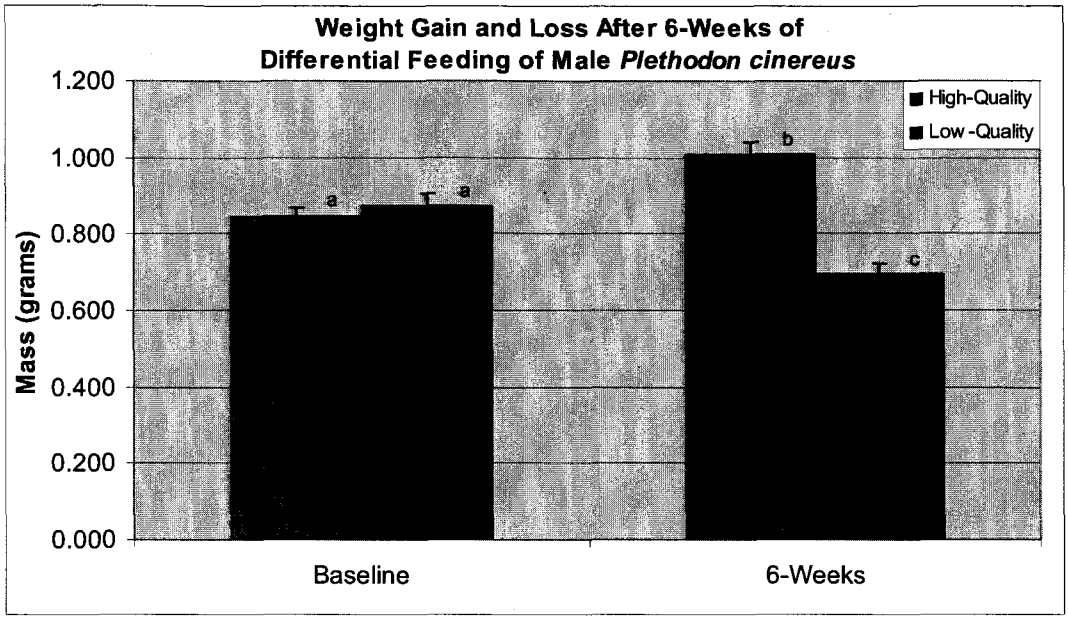

Figure 17. Weight differences for males fed different diets over a six-week period. All males were arranged into size-matched pairs prior to experimentation. Prior to differential feeding, there was no significant difference between the weights of the two diet groups (two-tailed T-test; $p>0.05$). After a six-week period, there was a significant difference between the weights of the different groups ($p<0.00001$). Comparing weight differences within groups: High-Quality males weighed significantly more after six weeks of feeding ($p<0.001$), while Low-Quality males weighed significantly less ($p<0.00001$). Error bars represent Standard Error.

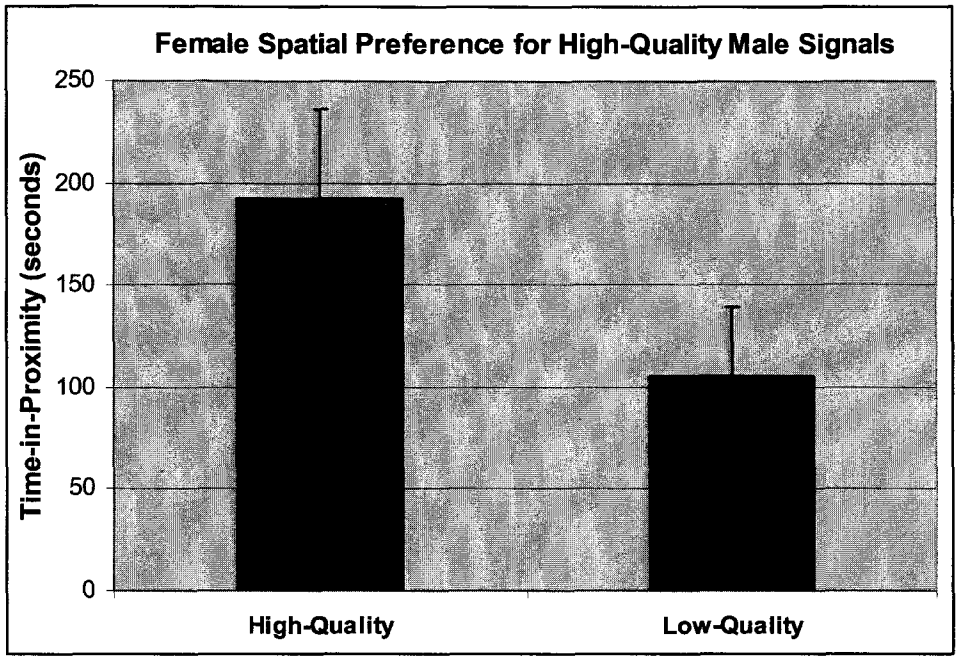

Figure 18. Time-in-Proximity of gravid females to the scents of High- and Low-Quality males after six weeks of differential feeding. While the general trend was in support of the hypothesis (and previous findings), females did not spend a significantly greater amount of time associating with the High-Quality scent (two-tailed T-test; p=0.13). This seems in contradiction to the hypothesis, but unexpected biochemical differences seem to have affected the data in this case, which further suggest the ability of females to distinguish between scents (see Discussion). Error bars represent Standard Error.

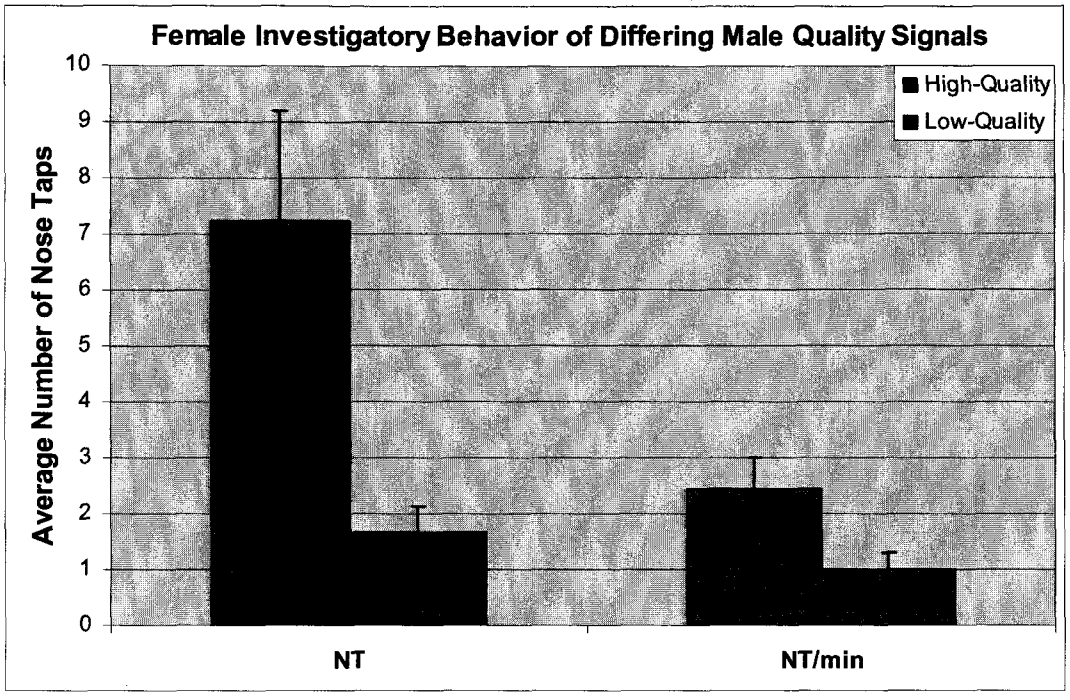

Figure 19. Number and rate of investigatory Nose Taps towards the scents of High- and Low-Quality males by gravid female. As predicted, females did exhibit a significantly greater number of Nose Taps to the scent of High-Quality males (two-tailed T-test; p=0.007), and also Nose Tapped at a significantly faster rate (p=0.03). This is in support of the prediction, as well as the findings of previous experimentation. Error bars represent Standard Error.

BCA Protein Assays: Field-Quality and Diet-Quality (MG + PCG)

Prior to a comprehensive protein assay with all samples, a control sample was run to verify that the AchCl/Ringer's extraction solution (in which the proteins were dissolved) did not affect the accuracy of the assay. The results of this control concluded that the solution did not affect the BCA assay (see Appendix C).

Field-Quality males demonstrated a large degree of variation in the amount of protein present in both the Mental and Postcloacal Glands (Table 1). Male Mental Glands ranged in protein concentration from 0.046-0.131 μg/μL, and seemed to be fairly well correlated with the weight of the source animal (Figure 20a). Postcloacal glands ranged in concentration from 0.362-1.594 μg/μL, and were even more strongly correlated with the weight of the sender (Figure 20b). Postcloacal samples tended to be an order of magnitude more concentrated, though this is not necessarily indicative of glandular concentration at the cellular level because the size of the gland region itself is larger, and therefore more skin is taken. See Appendix D for additional correlations of source-male characteristics and gland protein concentration including: TL, SVL and Weight/Length ratio.

When comparing gland protein concentration between Diet-Quality males (Table 2; Figure 21), High-Quality males had a significantly greater amount of protein in both the Mental (two-tailed T-test; df=17; t=3.6; p=0.0022) and Postcloacal Glands (df=17; t=4.1; p=0.0007). Data for each individual male pairing can be seen in Figure 22. In all but two cases in the MG sample (Pair 6, 8), the High-Quality male had a larger amount of protein present; there was only one such case in the PCG (Pair 8).

When correlating the relationship of gland protein concentration to the weight of the sender after six weeks of differential feeding, there are strong and consistent trends in which larger males (in terms of mass) have a larger amount of secretory proteins in both glands (Figure 23). In addition, as not all High-Quality males responded the same way to the diet (see Table 2; Figure 22), protein concentration of the two glands was also analyzed as a function of the energy-budget of the sender (Figure 24). Males on positive energy-budgets (gained weight over the six week feeding) had a significantly greater protein concentration than those on a negative energy-budget in both the MG (two-tailed T-test; df=14; t=5.1; p=0.0001) and the PCG (df=13; t=4.3; p=0.0008).

Protein Concentrations of Field-Quality Males (MG + PCG)

Animal #	SVL (mm)	TL (mm)	Weight (g)	[MG] (µg/µL)	Total (µL) in MG	[PCG] (µg/µL)	Total (µg) in PCG
48	38	70	0.677	0.049	9.744	0.545	109.071
45	38	74	0.681	0.046	9.163	0.362	72.493
46	39	61	0.611	0.067	13.349	0.464	92.798
47	39	67	0.697	0.059	11.721	0.829	165.805
33	40	67	0.675	0.099	19.744	0.369	73.837
32	40	76	0.732	0.046	9.279	0.425	85.034
34	41	59	0.685	0.074	14.744	0.277	55.324
35	41	69	0.795	0.124	24.744	0.799	159.833
69	42	72	0.826	0.086	17.186	0.563	112.655
58	42	74	0.883	0.071	14.279	0.589	117.880
61	42	75	0.882	0.074	14.860	0.935	187.005
70	42	76	1.000	0.131	26.256	1.028	205.518
50	42	81	0.990	0.104	20.791	0.853	170.582
53	43	77	0.878	0.083	16.605	0.834	166.701
57	43	78	0.852	0.100	20.093	0.788	157.593
52	44	80	0.938	0.092	18.349	0.950	189.991
66	44	80	0.999	0.096	19.163	1.056	211.191
39	44	83	1.005	0.105	21.023	0.944	188.797
54	45	82	1.070	0.081	16.256	0.816	163.117
36	45	85	1.080	0.113	22.651	0.869	173.867
64	45	85	1.120	0.124	24.860	1.594	318.835
56	45	86	1.040	0.101	20.209	0.931	186.259
Mean	42.00	75.32	0.869	0.088	17.503	0.765	152.918
StdDev	2.28	7.51	0.157	0.025	5.028	0.299	59.820
StdErr	0.48	1.60	0.033	0.005	1.072	0.064	12.754

Table 1. MG and PCG protein concentrations of Field-Quality males (no experimental manipulation). The source-animal sizes and weight can be seen on the left (sorted from smallest to largest), followed by the concentrations of the individual glands. For each gland (MG and PCG) there are values for the concentration of protein within the extraction solution (µg/µL), in addition to the total mass of protein present within the extraction solution (concentration x 200µL). This is representative of the total amount of protein secreted from the gland into solution during the extraction. Presented at the bottom are mean numbers, followed by Standard Deviation (StdDev) and Standard Error (StdErr).

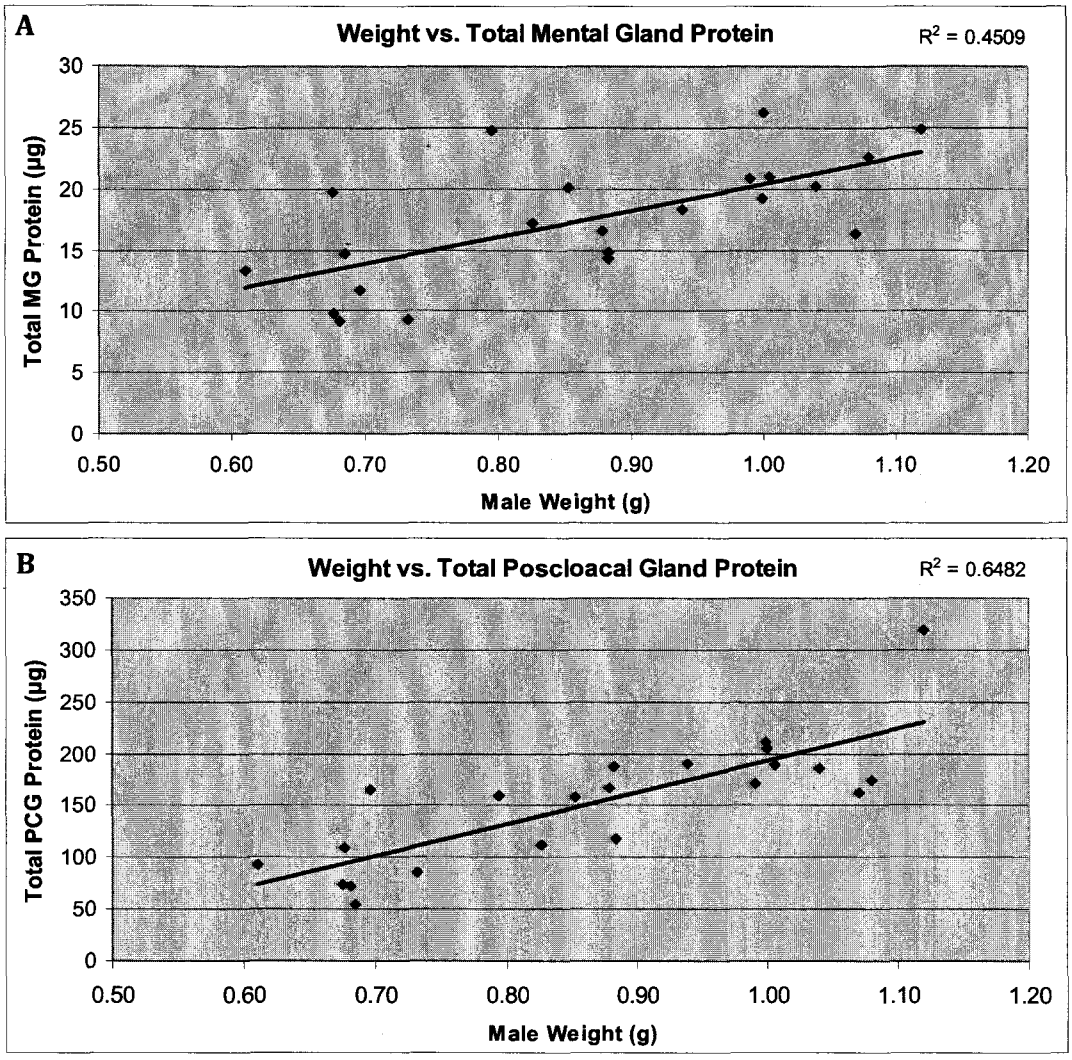

Figure 20. Correlational relationships with Field-Quality male weight and the amount of protein present in the Mental (MG) and Postcloacal Glands (PCG). In both cases, there is a clear linear relationship, with larger males possessing a larger amount of secretory protein. In the case of the MG, the relationship is less consistent (r^2=0.4509), while in the PCG the relationship is well conserved (r^2=0.6482). In both cases, there seems to be a valid trend.

Protein Concentrations of Diet-Quality Males (MG + PCG)

Animal #	SVL (mm)	TL (mm)	6-Week Δ Mass (g)	[MG] (µg/µl)	Total (µg) in MG	[PCG] (µg/µl)	Total (µg) in PCG
1-H	40	71	0.292	0.093	18.573	2.282	456.457
2-H	40	74	0.254	0.128	25.506	1.671	334.212
3-H	40	75	0.290	0.158	31.552	1.246	249.185
4-H	41	74	0.178	0.072	14.303	1.263	252.621
5-H	42	74	0.159	0.119	23.898	2.441	488.235
6-H	42	75	-0.087	0.043	8.590	1.200	240.024
7-H	42	79	0.281	0.119	23.842	1.323	264.645
8-H	43	74	-0.029	0.029	5.872	0.792	158.432
9-H	43	75	0.094	0.079	15.856	1.139	227.714
10-H	44	74	0.237	0.125	25.007	1.841	368.281
11-H	44	80	0.076	0.091	18.130	0.814	162.727
12-H	45	83	0.235	0.203	40.592	2.206	441.284
Mean	42.17	75.67	0.165	0.105	20.977	1.518	303.651
StdDev	1.70	3.31	0.127	0.048	9.612	0.563	112.545
StdErr	0.49	0.96	0.037	0.014	2.775	0.162	32.489

Animal #	SVL (mm)	TL (mm)	6-Week Δ Mass (g)	[MG] (µg/µl)	Total (µg) in MG	[PCG] (µg/µl)	Total (µg) in PCG
1-L	40	69	-0.124	0.059	11.862	1.223	244.605
2-L	40	74	-0.210	0.025	4.985	0.635	126.941
3-L	40	75	-0.032	0.058	11.530	1.149	229.718
4-L	41	74	-0.151	0.024	4.708	0.738	147.553
5-L	42	74	-0.224	0.041	8.202	0.400	79.990
6-L	42	75	-0.250	0.081	16.300	1.003	200.516
7-L	42	79	-0.194	0.026	5.262	0.516	103.179
8-L	43	74	-0.060	0.061	12.140	0.888	177.614
9-L	43	75	-0.258	0.029	5.762	0.429	85.715
10-L	44	73	-0.176	0.092	18.352	0.686	137.247
11-L	44	80	-0.211	0.015	2.933	0.308	61.667
12-L	45	82	-0.240	0.071	14.136	1.054	210.823
Mean	42.17	75.33	-0.178	0.048	9.681	0.752	150.464
StdDev	1.70	3.47	0.073	0.025	5.067	0.309	61.825
StdErr	0.49	1.00	0.021	0.007	1.463	0.089	17.847

Table 2. MG and PCG protein concentrations of High- (top) vs. Low-Quality males (bottom). The source-animal sizes and 6-week weight changes (Δ Mass) can be seen on the left. The animal number corresponds to the size-matched diet-pair (1-12) and the specific diet (H/L=High/Low) on which each individual of the pair was placed. For each gland there are values for the concentration of protein within the extraction solution (µg/µL), in addition to the total mass of protein present within the extraction solution (concentration x 200µL). This is representative of the total amount of protein secreted from the gland into solution during the extraction. Means for all categories are presented at the bottom, followed by Standard Deviation (StdDev) and Standard Error (StdErr).

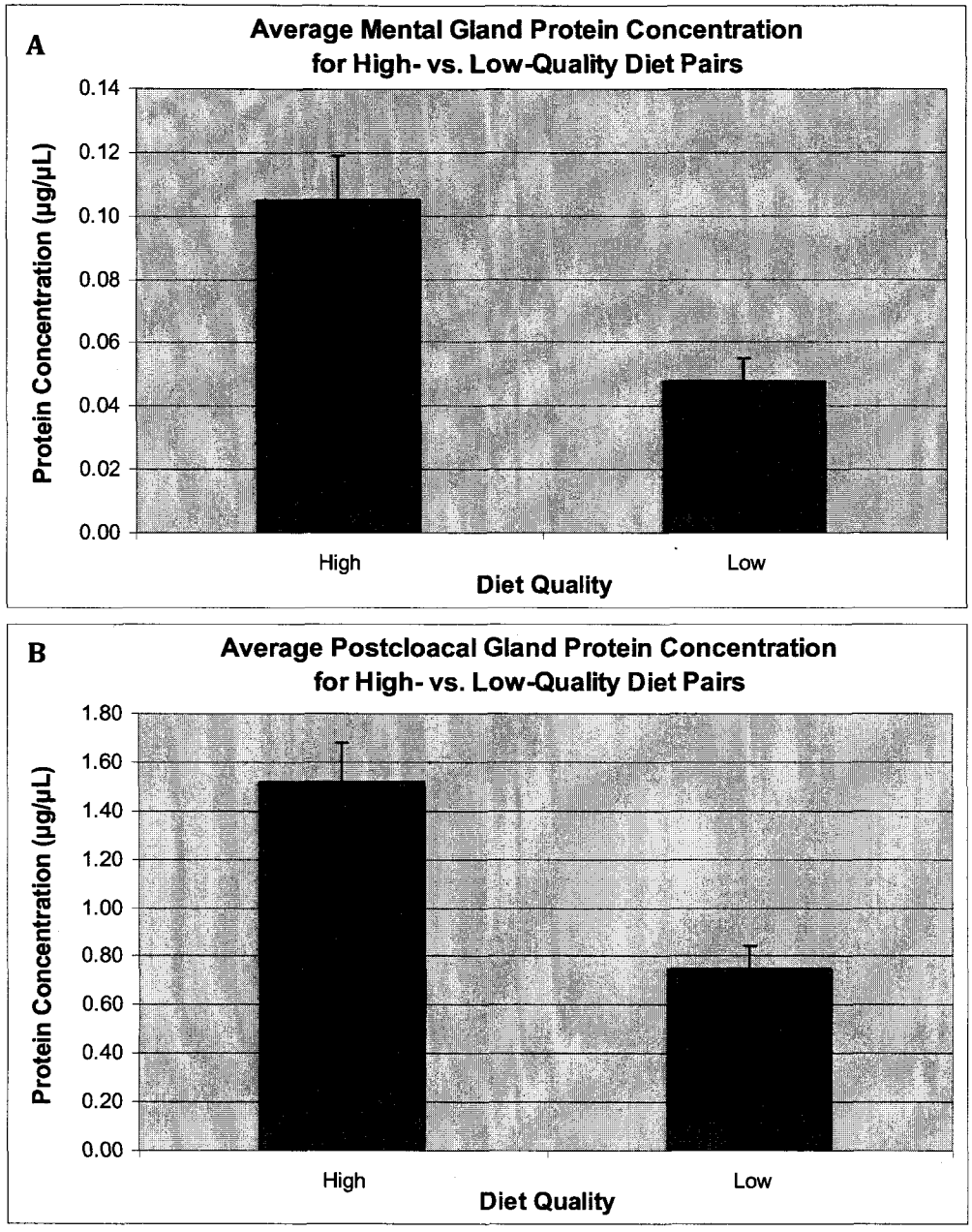

Figure 21. Average MG (A) and PCG (B) protein concentrations for High- vs. Low-Quality males. High-Quality males have a significantly greater concentration of protein in the MG (two-tailed T-test; p=0.0022) and PCG (p=0.0007). Error bars represent standard error.

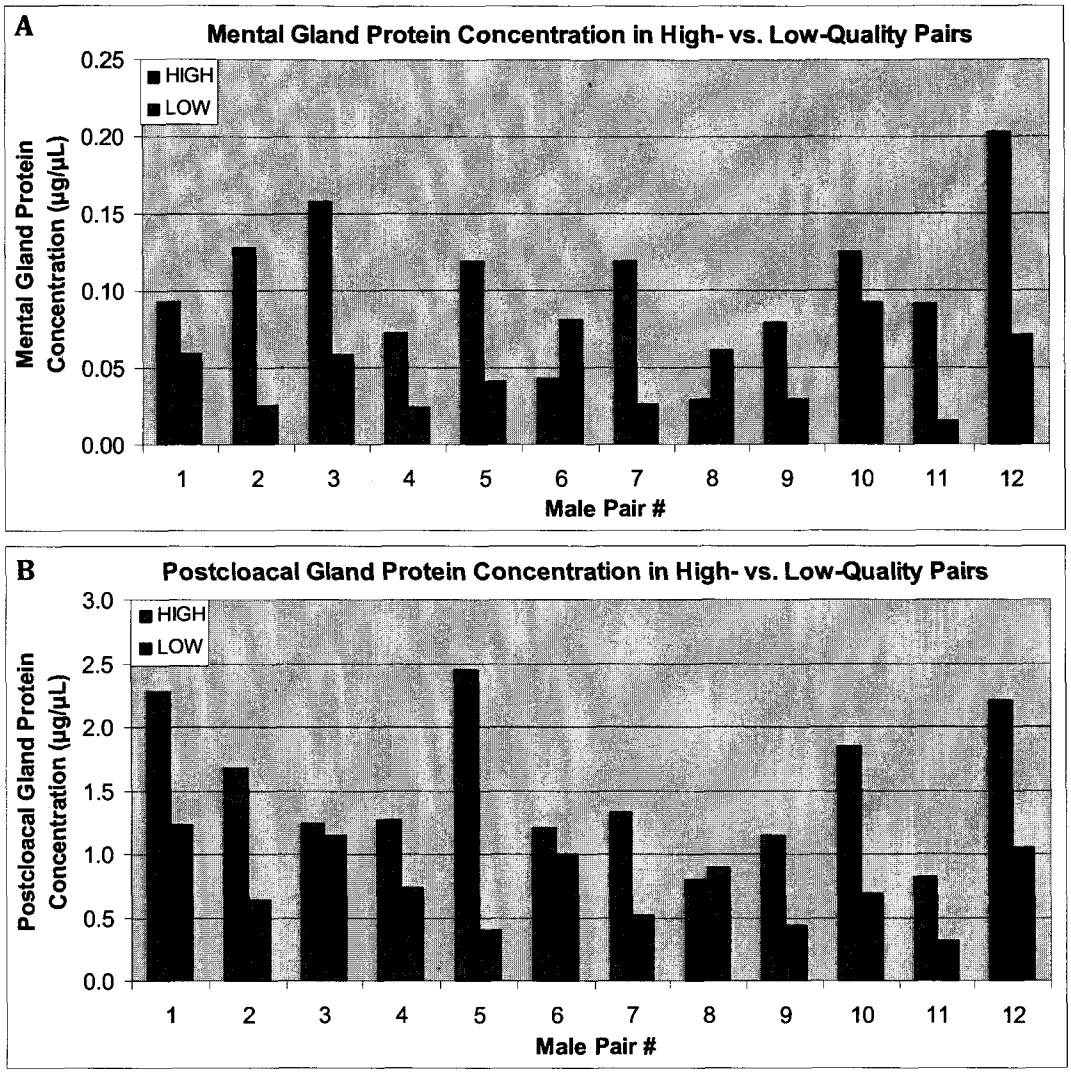

Figure 22. Individual analysis of MG (A) and PCG (B) protein concentrations between male High- and Low-Quality pairs. Males placed on High-Quality diets tend to have a larger amount of protein in both glands. However, there are two cases in which the Low-Quality male had more protein in the MG (Pair 6 and 8), and one case in the PCG (Pair 8). Between-treatment comparisons have revealed this to be a statistically valid trend (Figure 21).

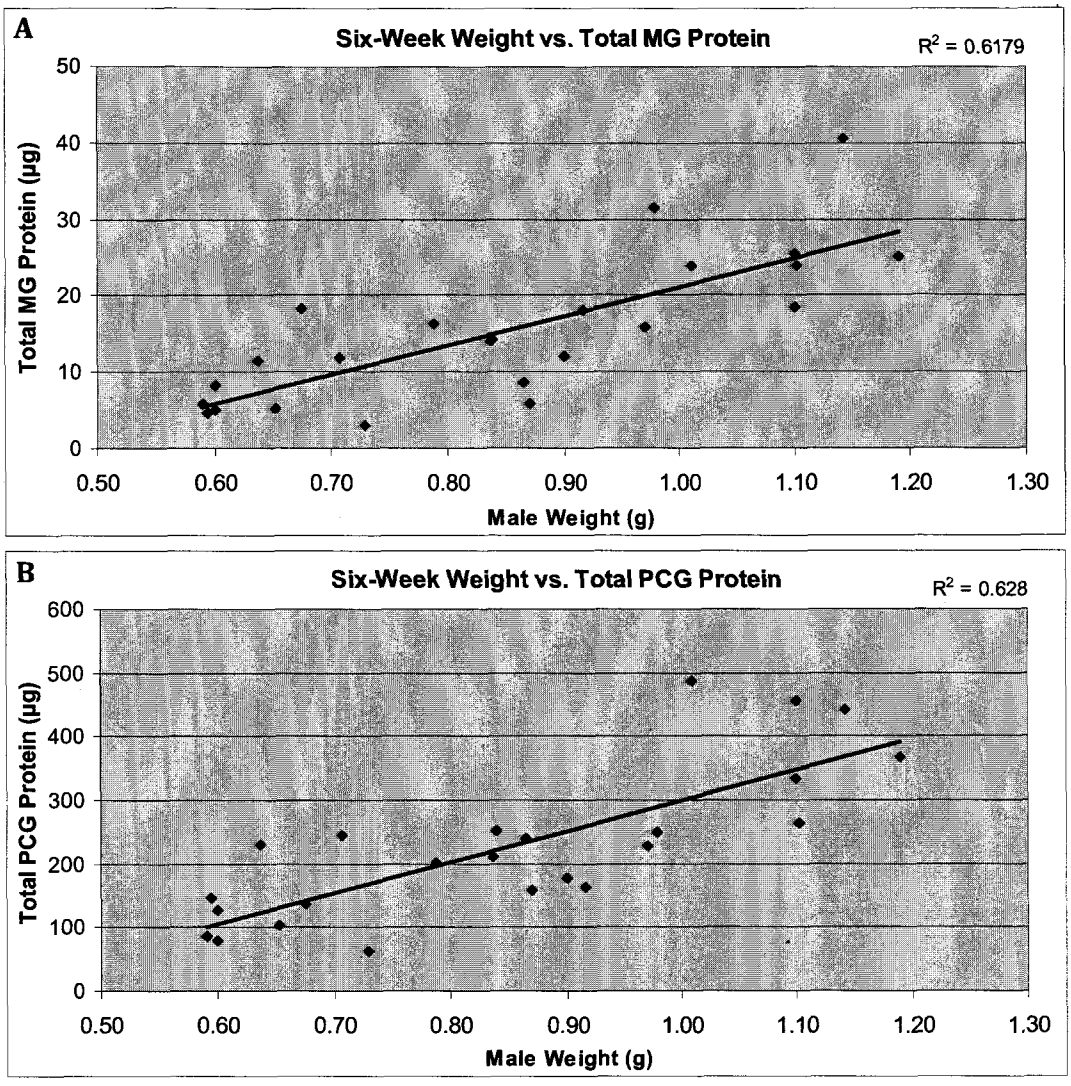

Figure 23. Weight of Diet-Quality salamanders after six weeks of differential feeding correlated with the protein concentration of Mental (A) and Postcloacal Glands (B). There seems to be a strong trend that larger (mass) males make more protein in both glands.

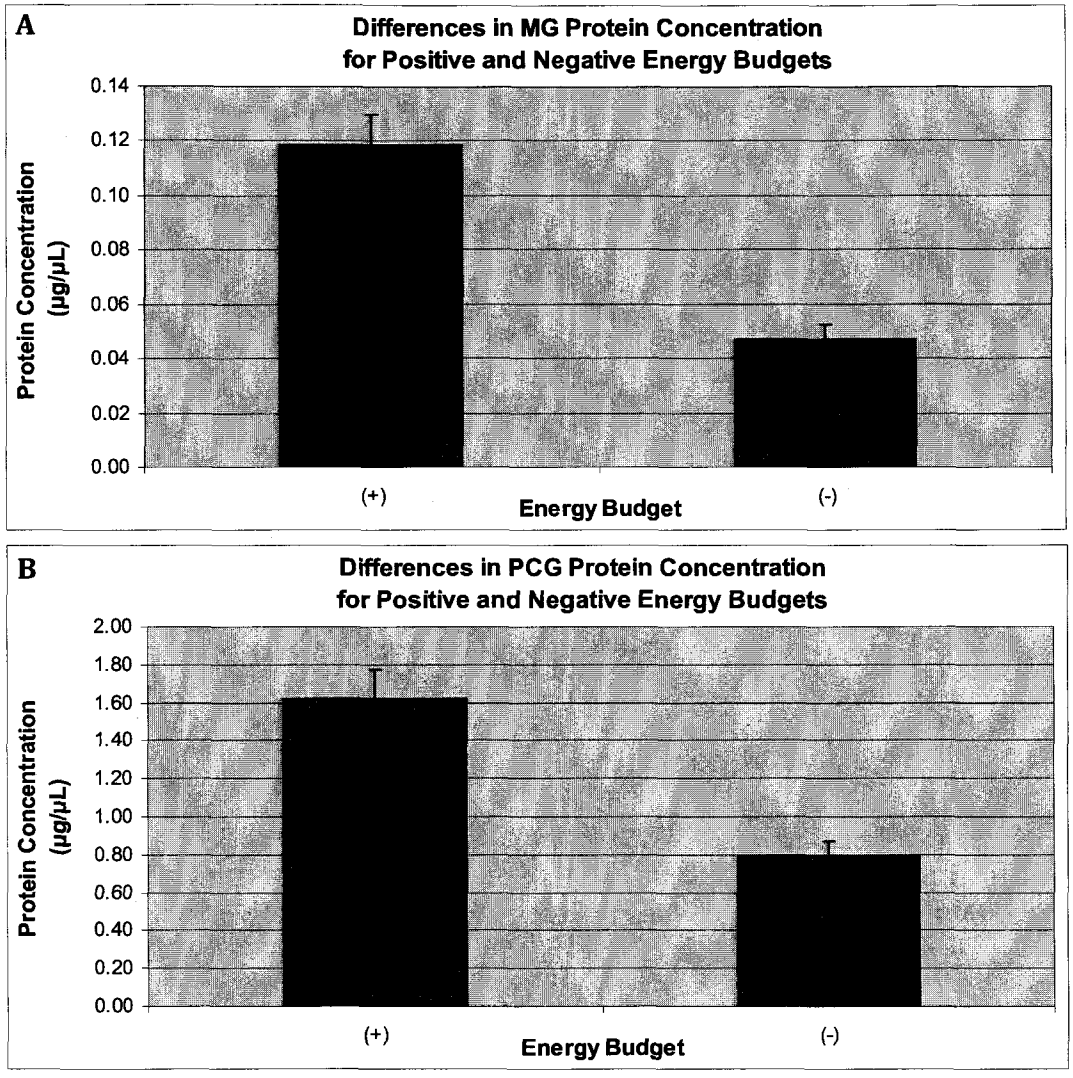

Figure 24. Protein concentrations of Mental (A) and Postcloacal Glands (B) as a function of energy budget (based on changes in weight over six weeks of differential feeding). As not all High-Quality individuals actually gained weight over the course of feeding, this analysis divides the sample into groups based on those who gained weight (n=10) and those that lost weight (n=14). Males on positive energy-budgets have significantly greater protein levels in the MG (p=0.0001) and the PCG (p=0.0008). Error bars represent standard error.

RP-HPLC Analysis of Field- and Diet-Quality Extracts

The Field-Quality RP-HPLC analysis of Mental Gland extract revealed an interesting shift in expression patterns from the preliminary analysis of Section II.B.2 (with extractions taken in November, at the very end of the breeding season). The Field-Quality baseline individuals, however, had extractions done during the peak at the onset of spring breeding (early May). The MG analysis revealed a drastic increase in a component that elutes at ~56 minutes (Figure 25). This is very similar to the elution time of PRF (Rollman et al., 1999; Feldhoff et al., 1999), as can be seen in the chromatogram of *P. cinereus* from Virginia (Figure 9). See Appendix A.7 for a full version of the Field-Quality MG chromatogram.

The differences between the samples from May (Field- and Diet-Quality) were different enough in key respects from the preliminary HPLC analysis of Section II.B.1 (November), that the terminology used to describe peaks from those runs will not be used. The labeling of peaks in that analysis was useful in describing overlap and consistency between runs within-sample, but the direct comparison to these samples proved less feasible. Thus, peaks of interest in the Diet-Quality sample are numbered for use in comparison. The variability between diet group samples was much lower; peak expression, as well as resolution, was consistent across High- and Low-Quality pooled samples (Figure 26). While there do not appear to be any obvious peaks that are present/absent in the different samples, there do seem to be changes between the relative proportions of certain peaks of interest (Figure 27).

The RP-HPLC analysis of High-Quality individuals encountered a problem with pressurization during the run, which caused the elution to stop after 30 minutes. The second half of the sample was then run after the column was "stripped" (to remove anything causing the build-up in pressure). The chromatogram displayed (Figure 26b; again in Appendix A.9) is a corrected version in which both runs were spliced together over common axes. The absorbance values and elution times were corrected for and are completely to scale across both High-Quality runs, but also compared to the Low-Quality run. Since the elution stopped at t=30 minutes, the elution time values above the peaks in the second half of the High-Quality chromatogram are therefore only correct when adding 30 additional minutes. Again, this was compensated so as to be correct in relation to the x-axis.

The Field-Quality PCG pooled sample (Appendix A.10) revealed far fewer peaks compared to the sample from the preliminary analysis (Appendix A.4). This sample possessed only proteins >3kDa, and thus reveals which peaks within the total mixture of the initial analysis meet those size criteria (Figure 28). These peaks occur at: 42.8, 43.9, 45.7 and 46.9 minutes. Unfortunately, the same problem of over-pressurization continued during the analysis of the PCG samples between diet groups, and data could not be obtained for these samples. This analysis will therefore be attempted in the future using more rigorous filtration methods to isolate the specific proteins in question, and avoid other contaminants that can clog the columns and cause over-pressurization. However, the resolution between PCG peaks after Ultrafiltration has greatly reduced the number of protein components within the overall mixture, making future experimentation much more plausible.

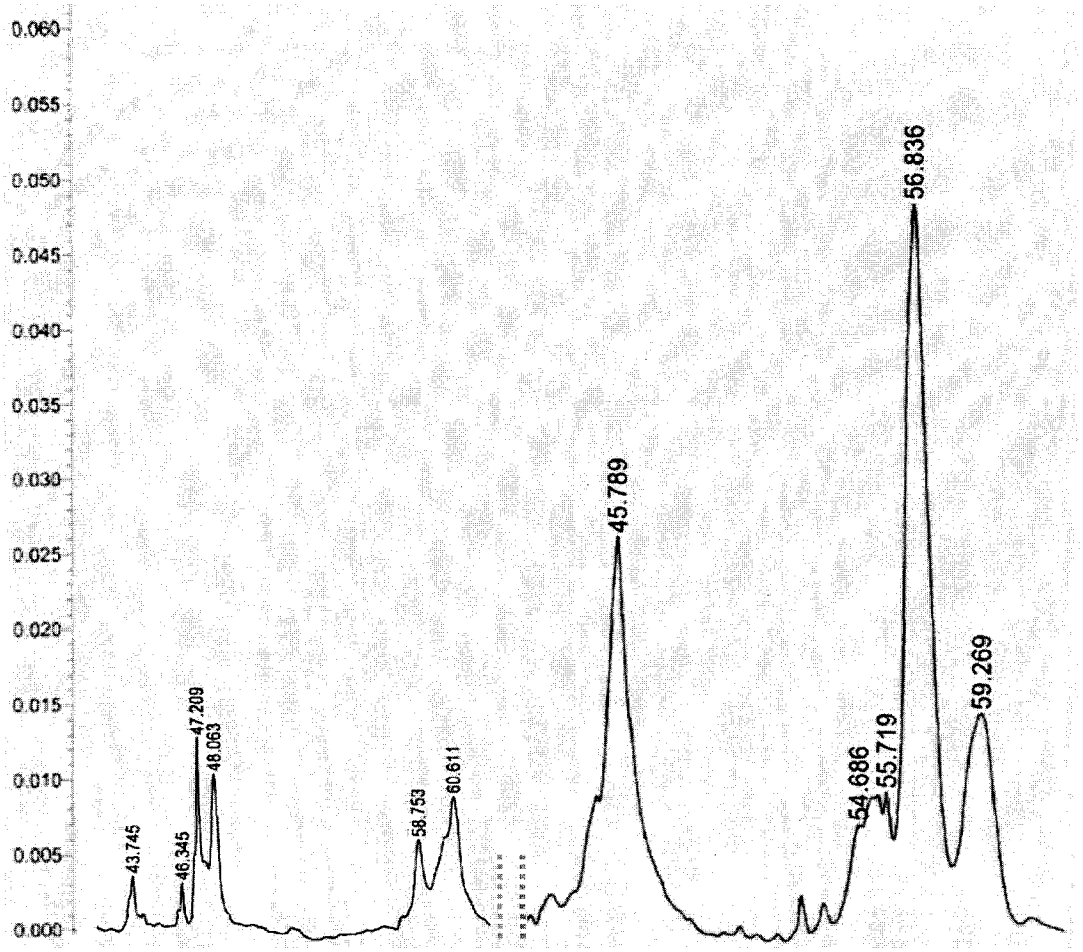

Figure 25. Overlay of Pooled MG Extracts from November (Section II.B.1; black line) and May (Field-Quality; red line). Amplitudes have been adjusted for direct comparison, although total amplitude is only indicative of the total protein loaded, which differed between runs. Thus, the relative comparison between which peaks are expressed is most important. Absorbance units can be seen as the scale on the left, and elution times can be seen above dominant peaks. There seems to be a shift in expression patterns between the two samples, although the variability between all MG runs was fairly high. There are a number of variables that may account for these differences (see Discussion).

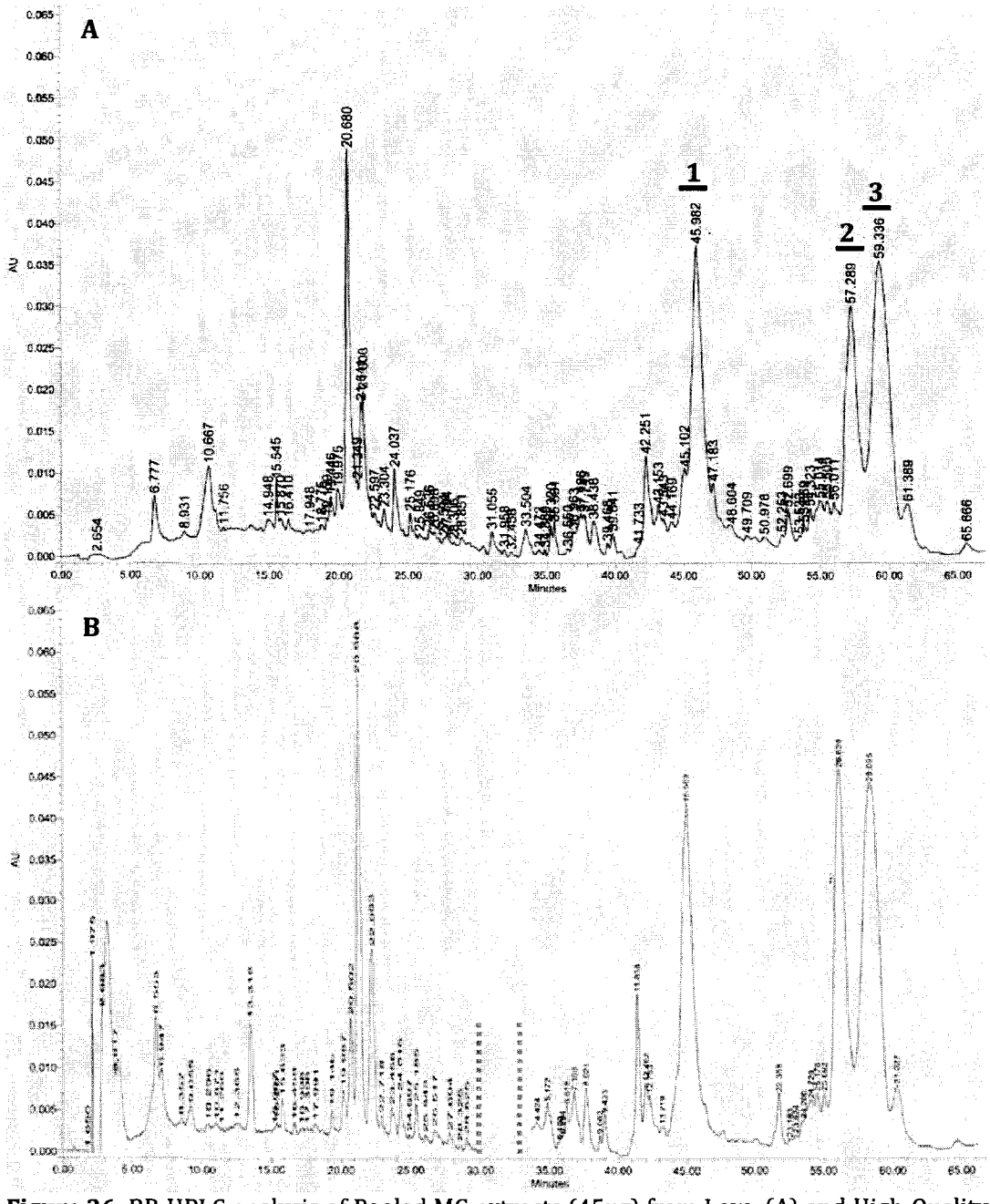

Figure 26. RP-HPLC analysis of Pooled MG extracts (45µg) from Low- (A) and High-Quality males (B). See Appendix A.8 and A.9 for larger chromatograms. The High-Quality analysis (B) was done over two runs because of pressurization problems within the column (t=30; dashed lines); the separate chromatograms were then overlaid, with elution times and absorbance values corrected for. There seems to be a shift in relative proportions of certain peaks in High-Quality individuals (peaks 1-3 indicate areas of potential interest). Peak #2 is within the range of PRF, and High-Quality individuals had a higher relative proportion of this component. High-Quality males therefore appear to have more putative PRF.

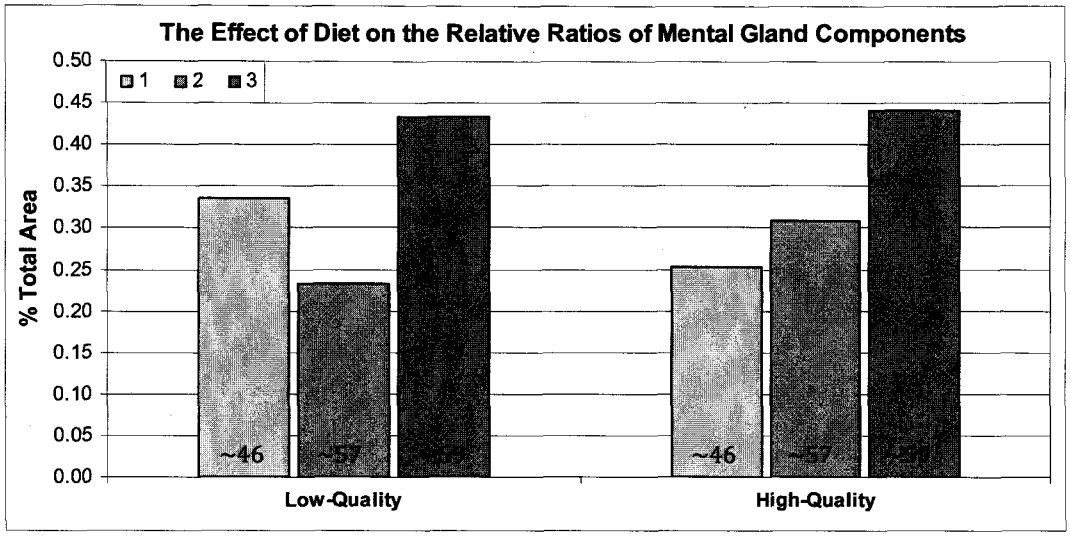

Figure 27. Relative ratios of individual dominant pheromone components between males on Low- and High-Quality diets. Peak numbers correspond to the chromatograms of each sample (Figure 26), and elution times of each peak can be seen at the base of the appropriate column. These peaks were focused on because they are particularly abundant, but also elute later from the column. High-Quality individuals demonstrate a higher proportion of component #2, however this can not be statistically verified in this analysis.

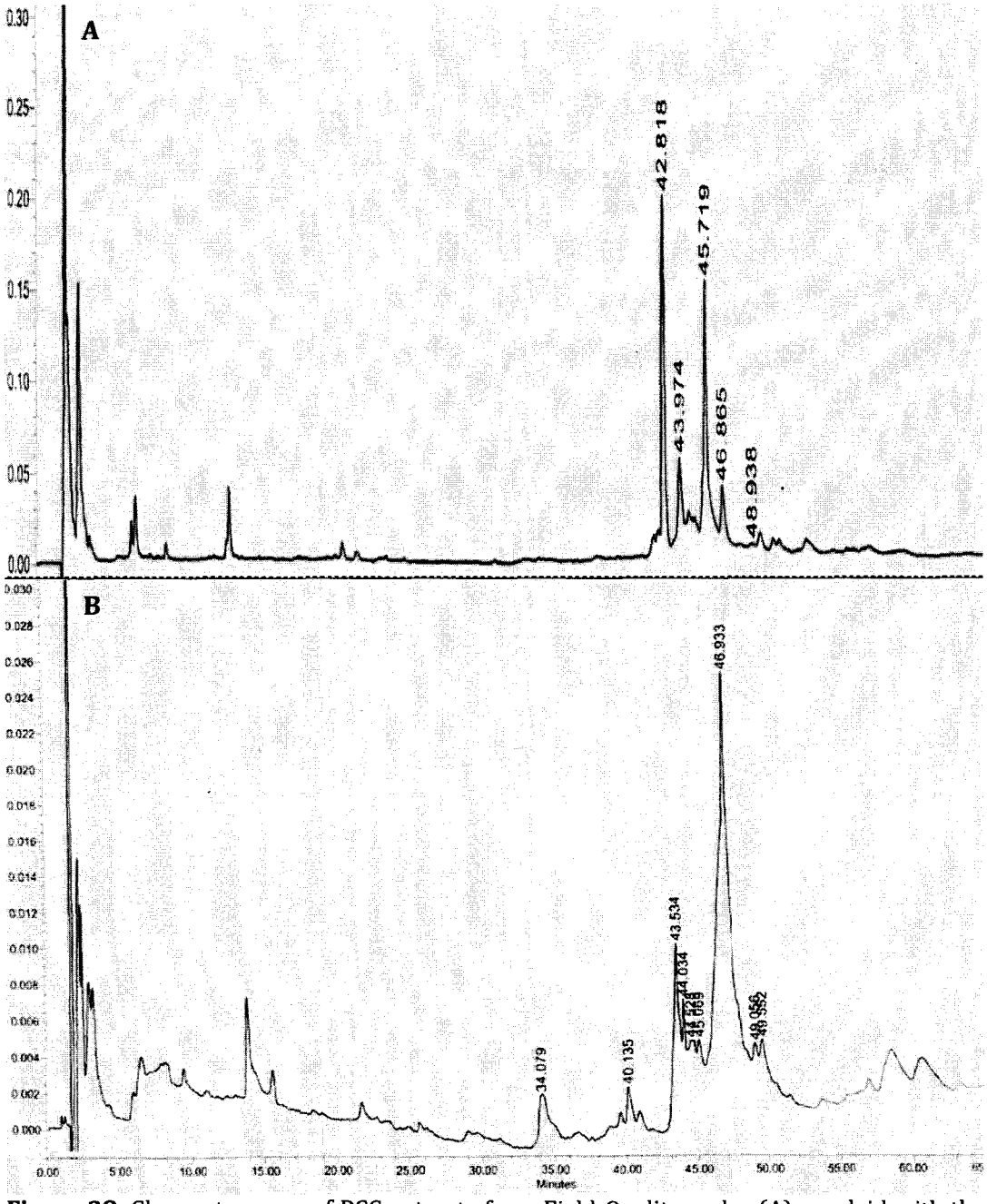

Figure 28. Chromatograms of PCG extracts from Field-Quality males (A) overlaid with the preliminary analysis from Section II.B.1 (B). The Field-Quality sample was subjected to a high-pass ultrafiltration (3 kDa). Thus, the top chromatogram (A) demonstrates the elution of only proteins that are greater than 3 kDa, in comparison to the total mixture of all molecules, including non-proteinaceous components (B). The range of absorbance units (y-axis) have been lined up between samples, but the Field-Quality sample has absorbance units an order of magnitude greater than the initial analysis (0.30 vs. 0.03 respectively).

Discussion

The Effect of Diet on Size and the Relationship to Total Protein Levels

The first set of results in this experiment revealed that the two diets did in fact have a profound impact on male physiology (weight gain/loss; Figure 17; Table 2), as well as the amount of secretory protein in both glands (Table 2; Figures 21-24). Male salamanders on High-Quality diets gained a significant amount of weight after six weeks of feeding ($\overline{X}$ = 0.165 g), while males on Low-Quality diets lost a significant amount of weight ($\overline{X}$ = - 0.178 g). Interestingly, in both cases there was a large degree of variability in the amount of weight gained or lost within treatment groups (Table 2). Some High-Quality males added ~40% of their initial weight after six weeks of feeding; others gained marginal amounts, while two individuals in the High-Quality group actually lost weight over the feeding period. There was similar diversity in the Low-Quality group between individuals (though it was less varied than the High-Quality males): some animals lost ~30% of their initial weight, while others lost only marginal amounts.

The feeding protocol provided a relatively constant amount of food within treatment groups, so this diversity in weight gain/loss must be accounted for by other factors. These factors are likely to be individual foraging rates and/or abilities (as observed by Jaeger, 1980a), but metabolic differences between individuals are also likely to be highly variable. Jaeger (1980a) identified distinct differences in the energy-budgets of individuals in both wet and dry periods (as prey availability is different between these two degrees of rainfall). Thus, regardless of any other

factors (such as territoriality), foraging abilities seem to be drastically different between individual salamanders. As the animals were housed in relatively small containers (15 x 15 x 5 cm), it seems highly unlikely that animals would not have been able to identify and locate prey items. Thus, individual metabolic demands may modulate the degree to which a salamander feels inclined to feed.

In any case, individual males seemed to differ a great deal in the amount of weight change over the feeding period, but greater weights were always strongly associated with a greater amount of secretory protein. In Field-Quality males, larger animals always had a greater amount of secretory protein in the MG and PCG samples (Figure 20). This was also observed with the Diet-Quality sample (Figure 23). Interestingly, regardless of the initial weight of the sender, the energy-budget seemed to demonstrate the same trend: individuals that were gaining weight have a greater amount of secretory protein in both glands than individuals that were losing weight (Appendix D.4). This is not surprising considering the constant demand for the production of scent-mark signals. Territorial animals are obligated to maintain an adequate amount of pheromone for territorial advertisements, as these markings strongly influence the ability to maintain the territory (Jaeger, 1986; Jaeger et al., 1986). Simons et al. (1999) also described a constant state of release and replenishment of secretory granules in the glands of the integument (also observed by the PCG histology of Section II.E). These secretory glands are constantly replenishing their products, requiring the transcription and translation of the appropriate messages. All of this takes energy, and if an individual acquires less

energy through their diet than a conspecific, they will have less energy available to dedicate towards producing these communicative molecules.

The concept of great importance in this situation is that the energetic demand for renewal of pheromone products provides relatively instantaneous input regarding the relationship between the source animal and the environment. The energetic condition of the sender depends on the resources available to him (his territory-quality), as well as his own foraging ability, digestion efficiency and metabolic condition. Regarding the former, the resources available to the sender also provides indirect information about the competitive ability (RHP) of the source animal in order to obtain the high-quality territory and successfully defend it. Again, the ability to successfully defend a territory depends a great deal upon the ability to scent-mark (Jaeger, 1986), and thus the relationship between foraging, territoriality and scent-marking has come full circle. All of these essential requirements are highly interrelated, and all of them will obviously have a large impact on the reproductive fitness of the individual.

Returning to the experimental data, the BCA protein assays of Diet-Quality males supported the prediction that High-Quality males would have more protein present in the secretory glands (Figure 21). However, when viewing the data for each individual, the variability within treatment-groups is again quite large. Just as males within treatment-groups differed in their degree of weight change, and as weight and energy-budget have been shown to be directly proportional to the amount of secretory protein (Figure 20; 23; Appendix D.3 and D.4), it is not surprising that there was also a high degree of variability in the amount of secretory

protein present (Figure 22). In almost all cases, High-Quality males had more protein, but there are a few abnormalities: one pair (#8) had a Low-Quality male with more protein in both the MG and PCG. As could be expected (based upon the relationships between weight, energy-budget and protein levels described above), this individual (#8-H) was one of the two High-Quality males that lost weight over the feeding period, the other being # 6-H. This individual (#6-H) also had less MG protein than his corresponding Low-Quality partner; they were also very close in PCG protein levels. In summary, only two High-Quality males lost weight over the feeding period, and these were also the only two cases in which their Low-Quality partner had more MG protein, and the only one case in which the Low-Quality male had more protein present in the PCG.

Essentially, it seems that the current energy-budget of the sender is of tremendous importance to the amount of secretory protein present. In recognizing these abnormalities, the data was reanalyzed as a function of energy-budget, regardless of diet (Figure 24). Not surprisingly, there was a tremendous difference in the amount of secretory protein between groups, and as expected these differences were even more significant than when the data was analyzed between diet-groups (of which a few individuals did not respond like the rest, diluting the data). This analysis (Figure 24) was not intended to replace the initial analysis (High- vs. Low-Quality diets; Figure 21), but done to revaluate the data in light of the identified outliers. As the differences in protein levels were greater when analyzed by energy-budget (than by diet-group), it demonstrates that the few individuals that

did not respond the same way to the diet were still normal within the broader trend of energy-budget having a large input to the amount of secretory protein present.

Having acknowledged the individual abnormalities in weight change and gland protein, the female preference data can now be fully addressed. The female preference experiment was designed to serve as a control, to verify that the same trend previously observed (Section II.A.2) was occurring within the specific sample to be tested for biochemical changes. It can be positively concluded that this is in fact the case, based upon the tremendous differences in investigatory behavior observed (Figure 19). Females demonstrated a great deal more Nose Tapping to the High-Quality scents, but also Nose-Tapped at a faster rate (NT/min; compensated for the amount of time spent on each scent). These differences alone demonstrate that females were inferring stark differences between the two scents, but the spatial preference data (Figure 18) is not actually statistically significant. When investigating those data some interesting conclusions can be drawn, as they correspond to the individual anomalies in protein levels (Figure 22).

The female Time-in-Proximity data were quite well-conserved across all trials (with the preference being obviously for High-Quality scents), but there was one case in which a female made a substantial preference for the Low-Quality scent (for pair #8). This one data point alone was solely responsible for the lack of significance (when omitted, the two-tailed p-value = 0.03). Interestingly, this one female outlier is also the only case in which a Low-Quality male had a greater amount of PCG protein (Figure 22), demonstrating that the female was actually behaving appropriately according to the hypothesis that they can distinguish the

amount of protein present. When re-analyzing the data for female spatial preference according to the amount of protein present (regardless of diet-group), the result is that females do spend a significantly greater amount of time associating with scents containing more PCG protein (two-tailed T-test; $p = 0.017$). Essentially, regardless of why the males responded how they did to the diets, it appears that the relative High- and Low- quality of this particular pair were flipped to the focal female, and she responded accordingly, demonstrating a clear preference for the scent of the male with more PCG protein. When coupled with the overwhelming difference in investigatory behavior, this provides clear evidence that females were differentiating between the qualities of the source individuals.

The first hypothesis of this experiment (regarding the total protein level) was supported, and the data obtained also provided some unforeseen insight into the remarkable ability of receivers to infer the quantity of protein present and differentiate between relatively small differences in protein concentration (when observed on an individual-basis, as the focal females themselves would be tested). The summation of all these data provides strong evidence for the use of the total protein level as a graded signal for male quality.

This may be the primary mechanism through which mate-choice and territoriality are facilitated (though changing isoform ratios will be discussed later). A larger number of proteins will have a greater chance of maximizing the activation of the appropriate sensory receptors, and the response in the receiving individual will be stronger. Eliciting as great a neural response in the receiving individual will certainly be of the utmost importance in the case of courtship pheromones

(PRF/PMF), as these proteins are known to play a role in increasing female receptivity and persuading her to complete the courtship ritual (Houck and Regan, 1990; Houck et al., 1998; Rollman et al., 1999; Wirsig-Wiechmann et al., 2002; 2006). Similarly, higher protein levels will also aid a great deal in territoriality: not only will a greater number of territorial-advertisement molecules elicit stronger responses in intruding individuals, but it will also lead to an increase in the active range and fading time of the signal (*sensu* Wilson, 1975). Both of these characteristics will allow for a more efficient and effective territory-defense.

For use in both courtship and territoriality, the energy required for protein production enables the differing protein levels of individuals to serve as a graded signal conveying exactly that: how much energy the sender has available to invest into chemical signaling. The size of the animal *per se* is not necessarily the only factor of interest to the receiver, but also the indirect information that can be inferred as a result of *how* and *why* the animal became so large* (as discussed above regarding the relationship between foraging, energy-budget and territoriality). While it has been definitively shown that the amount of secretory protein is responsible for conveying aspects of mate-quality, relative protein ratios could also be responsible for conveying different aspects of variable information. This, the second hypothesis, will now be specifically addressed.

* This is obviously not to say that these behavioral association choices are cognitively processed by these animals, but rather based upon instinctive behavioral patterns crafted by natural selection over long periods of time.

Further Biochemical Analysis and Relative Protein Ratios

The first finding of interest in the second RP-HPLC analysis (of Field-Quality males) was a shift in Mental Gland expression patterns from the preliminary analysis (of Section II.B.2). The preliminary analysis revealed no peaks in the PRF range, in the NH sample (though this is not true for the VA sample). The analysis of the Mental Gland pool of Field-Quality individuals (also from NH) revealed large peaks in the PRF range (Figure 25). This peak is not necessarily PRF, as that has not been confirmed through proteomic analysis, but it does provide strong initial evidence for its presence in the NH sample. The difference between these two elution profiles is likely due to the fact that the preliminary sample was obtained from animals that had been housed in the lab for several months, which could have caused the Mental Glands to revert to the non-breeding state (as per Pool and Dent, 1977). Also, the glands used in the preliminary sample were removed in November, and while this is technically in the breeding season (see Section I.A.2), it may be that the glands were not as hypertrophied as the Field-Quality males, whose glands were obtained in May. Overall, it seems that when samples from NH were obtained in May, with very little laboratory housing time (5-11 days), there are in fact peaks present in the PRF range.

The analysis of relative pheromone ratios in *P. cinereus* proved to be difficult for technical reasons with the HPLC. While the initial intention was to run each male's sample individually, the MG had to be run as pooled samples for High- and Low-Quality diets, and the PCG sample could not be run. The High-Quality sample (run second) encountered an aborted run due to over-pressurization of the column.

The second half of the sample was able to be run after the column was stripped, and the chromatogram was overlaid and corrected for retention times and absorbance units. The resulting comparison of High- and Low-Quality Mental Gland profiles (Figure 26) was a surprising salvage considering the technical difficulties encountered.

There were noticeable differences in relative pheromone ratios in the MG of *P. cinereus* between High- and Low-Quality individuals, though this could not be statistically verified because the samples were run as a pool (and therefore n=1 for each treatment). Even so, the analysis of the pooled sample provides the sum total of all individuals for each peak, and there can still be some conclusions drawn. The High-Quality individuals had an increase in the relative proportion of the peak eluting at ~57 minutes (peak #2 in Figure 26). This peak is within the range of PRF, and thus it seems that High-Quality males had an increase in the putative PRF (Figure 26; 27). The inability to run each sample individually, as well as a lack of knowledge of the exact identity of the *P. cinereus* peaks, led to the further analysis of PRF isoform ratios in *P. shermani* (Section II.D).

The analysis of the PCG was unfortunately limited because of continuing problems with the HPLC. The first sample run was the Field-Quality sample (see Appendix A.10). This sample was run through a 3 kDa ultrafiltration, and this allowed for a clearer perspective on which peaks in the preliminary analysis were from proteinaceous components. These differing chromatograms are overlaid for direct comparison (Figure 28). Unfortunately, the PCG samples between High- and Low-Quality individuals could not be analyzed due to the aforementioned technical

difficulties with the reverse-phase columns. An initial insight can be obtained from the SDS-PAGE comparison of the two polarities of the size-distribution of the Field-Quality sample (Figure 16; Section II.B.2), though this is not individuals on experimentally-manipulated diet. One band in particular (between 14-20 kDa) appears to be more prevalent in larger males, though additional experimentation is needed to conclude more distinct changes in pheromone profiles.

Despite methodological obstacles, there seems to be initial evidence for differing ratios within the pheromone mixture itself between source males with different characteristics. Again, because of the difficulties encountered in these analyses, a further study was carried out with the model system in which these methods have been fine-tuned (*P. shermani*). This allowed for the analysis of known pheromone components as they relate to males of differing sizes (Section II.D).

II.D. SIZE-OF-SENDER MENTAL GLAND ANALYSIS (*Plethodon shermani*)

Objective

One of the original goals of this research was to investigate how changes in qualities of the source-individual could be associated with specific changes in the biochemistry of the pheromone mixture itself. It was hypothesized that not only could the total volume of the signal be different between individuals and change over time, but also that the relative volumes of specific pheromone components could fluctuate as a function of physical and/or environmental characteristics. One of the specific interests was the Mental Gland of *Plethodon cinereus*, because a large volume of behavioral work has elaborated on the social function of these molecules, which could very well be modulated by environmental conditions. It has also been shown that eastern *Plethodon* species share two common components of this gland: PRF and PMF (Palmer et al., 2005; 2009; Kiemnec, 2009), and that both of these proteins exist in numerous isoforms (Rollman et al., 1999; Palmer et al., 2007a).

An initial attempt was made to identify changes in isoform ratios of Mental Gland components using *P. cinereus* as a model system. However, the biochemistry of the Mental Glands tested appeared to be far more different from *P. shermani* than previous research had alluded to. There were also stark contrasts between different populations of the same species (*P. cinereus* from NH vs. VA; separated by more than 700 miles). While the system initially envisioned for investigating this question proved to be less practical, the question remains one of great importance. Thus, the original model system for exploring the biochemistry of pheromone components was used to investigate the same objective; PRF could be reliably and consistently

identified, and the resolution between isoforms had been optimized through previous methodological development. Specifically, Mental Glands of *P. shermani* males were analyzed for the relative proportions of PRF isoforms, in order to identify any changes in the ratios associated with the size of the pheromone sender.

Methods

Male red-legged salamanders (*P. shermani*; n=20) were collected from Macon County, NC in August, 2009. Animals were housed at 16°C (14:10 L:D cycle) at Highlands Biological Station (Highlands, NC). Males are easily identified by the large protruding Mental Glands that distinguish them from females. The following day, males were sorted into two groups based on obvious size differences (Large and Small). Each animal was anesthetized in 7% ether, weighed (g), measured (SVL, TL; mm) and had their Mental Glands surgically removed. Upon removal, glands were immediately placed into 200 μL of AchCl extraction solution for one hour. At this time, glands were removed and the extraction was centrifuged (14,000x) for 10 minutes, after which the supernatant was removed and re-centrifuged. This process was repeated for a total of three rounds of centrifugation. Glands were then frozen at -80°C and brought back to the lab of Dr. Richard Feldhoff (Lousiville, KY) for RP-HPLC Analysis.

Extracts for each individual were tested for protein concentration through a BCA protein assay. This concentration was used to calculate the amount of volume needed to load a standard mass of total protein (25 μg) into a C18 RP-HPLC column (see Section II.B.1. for specifications). Protein concentrations of each individual's extract were also related back to the weight (g) of the Mental Glands from which

they were secreted. Samples were run via RP-HPLC for each individual, and the total absorbance area under each elution peak was integrated. This provides a comparison of the volume of each molecule eluting from the column at a given time. The objective of this study was to focus on relative ratios of PRF isoforms (B, C1 and C2) as associated with the size of the source-animal, and thus the absorbance values for only those peaks were totaled, and the percentage of each isoform (of the total PRF integration area) was calculated. A second comparison utilized the same analysis, but in relation to a third (non-PRF) component (C3). In this case the total of all PRF isoforms was compared to the area of the C3 component. Essentially this investigates the PRF:C3 ratio, using the total of PRF and C3 to calculate the relative proportions of each component to the other. In both cases, the percentages of each component were compared between Large and Small individuals, and analyzed with a two-tailed T-test assuming unequal variance.

PMF was not analyzed at this time because of the extreme diversity in number, proportion and structure of isoforms (n>50 isoforms; Feldhoff et al., unpublished data). This degree of variability is not currently feasible to investigate through the use of RP-HPLC alone.

Results

RP-HPLC analysis of male *P. shermani* produced a consistent and well-resolved separation of Mental Gland components, as expected. A characteristic chromatogram (Figure 29) clearly demonstrates the identification of the major pheromone components. The three isoforms of PRF elute latest of all with retention times of: ~51 (B), ~54 (C1) and ~55 (C2). Conversely, PMF elutes relatively early: ~30-40 minutes. The drastic amount of structural variability of the PMF isoforms is clearly demonstrated, with a large number of peaks within the elution range. Interestingly, this variability is even underestimated, as the differences in structure between some variants are too slight to be separated by RP-HPLC (Feldhoff, pers. comm.). The last of the major components of the *P. shermani* Mental Gland is the non-PRF protein C3, which elutes at ~41 minutes. These peaks are well-resolved and easily identified across multiple runs, and thus comparison between all individuals is feasible (see Appendix A.11 and A.12) for all *P. shermani* size-analysis chromatograms). Two individuals (Small # 1 and 3) had practically no protein present (via BCA assay), and were therefore not run through the RP-HPLC.

When comparing the relative proportions of individual PRF isoforms between Large and Small individuals (Figure 30), there were no significant differences between groups for isoform B ($df=15$; $t=0.9$; $p=0.37$), C1 ($df=13$; $t=0.5$; $p=0.59$) or C2 ($df=16$; $t=1.7$; $p=0.10$).

When analyzing the proportion of (non-PRF) C3 (Figure 31), there was a significantly higher proportion of C3 in Large animals than Small animals (two-tailed T-test; df=10; t=2.3; p=0.04). While this was significant through a between-group statistical analysis, when analyzed as a function of the actual weight of the sender, an interesting trend appears (Figure 32). Larger animals do have a higher proportion of C3, but the trend of the small males demonstrated an inversion of this, with the smallest males producing C3 proportions similar to the largest males. Thus, over the entire weight gradient, there seems to be a U-shaped curve; the largest and smallest individuals of the total sample demonstrate the highest proportion of C3.

Animals were also analyzed for the amount of protein present (Figure 33). Large males did have significantly higher concentrations of secretory proteins (two-tailed T-test; df=15; t=3.1; p=0.006). The size of the gland itself was also compared to the concentration of protein secreted (Figure 34). As expected, larger glands produced higher protein concentrations; this seemed to only be true to an extent, with the curve leveling off for the largest gland weights.

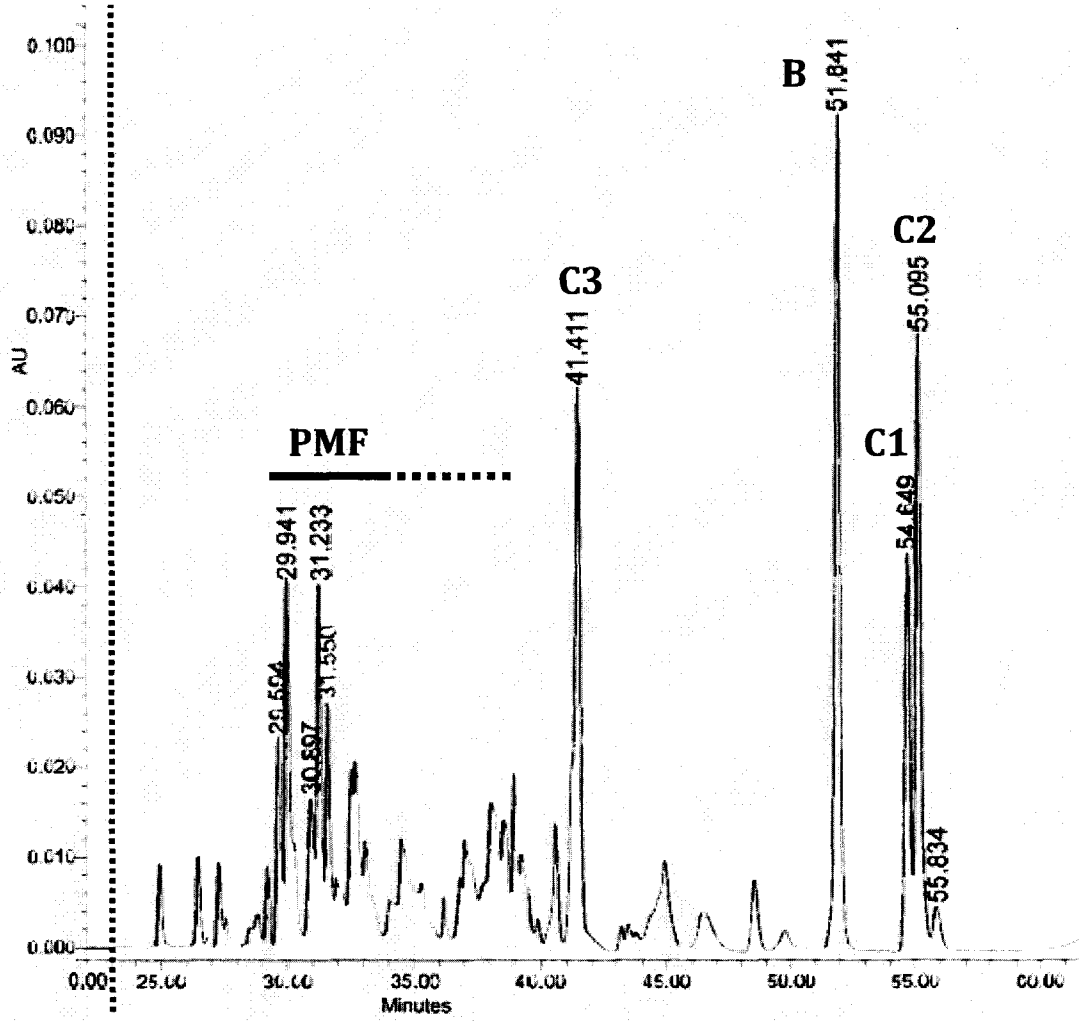

Figure 29. Representative chromatogram of Mental Gland extract from *P. shermani* males (Individual: Large #5). Elution times can be seen at the top of each dominant peak, along with labels of known components. PRF isoforms (B, C1 and C2) elute at: ~51, ~54 and ~55 minutes respectively. PMF isoforms elute in the 30-minute range; the great amount of structural variability is easily visible. The dashed line over the later eluents in the PMF range simply represent that the exact boundary of PMF isoforms is unclear. The non-PRF protein C3 elutes at ~41 min., and is by far the next most abundant component.

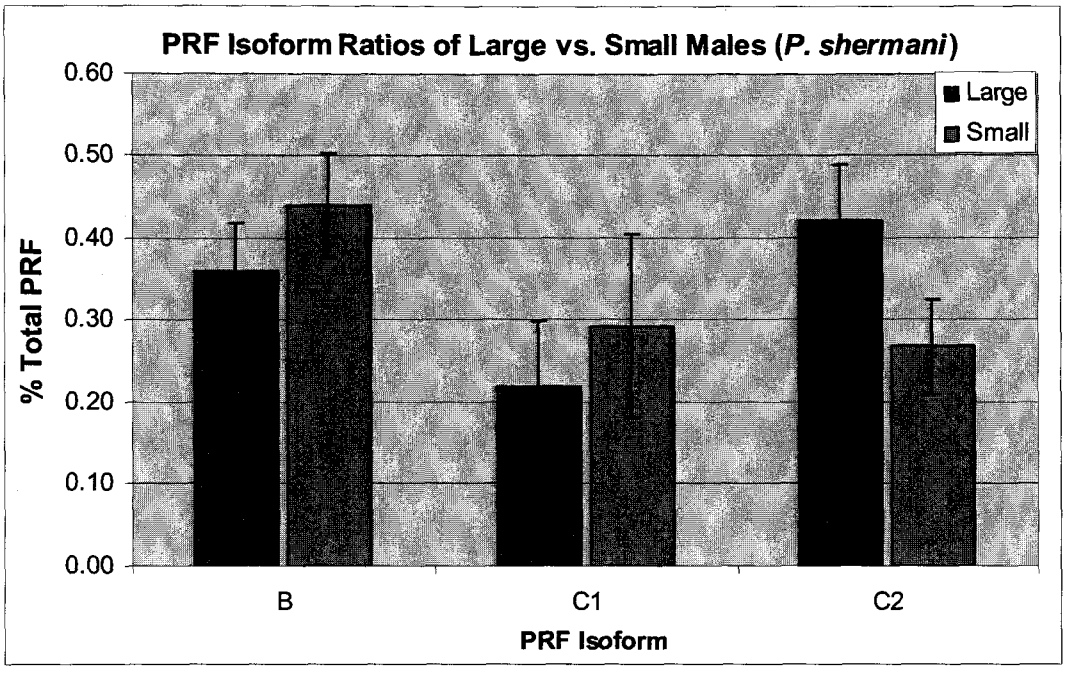

Figure 30. Relative proportions of individual PRF isoforms between Large and Small Red-legged salamanders (*P. shermani*; n=18). The y-axis is the percentage (0-1) of the total PRF area that each isoform constitutes. Relative proportions were calculated by combining the sum integrated area under all PRF peaks, and dividing the area of each component by the total. There is no significant difference in relative proportions of any of the isoforms between Large and Small males (two-tailed T-test; $p>0.05$). However, C2 does show an interesting trend that approaches significance (one-tailed $p=0.0502$), with larger males having slightly higher C2 percentages. Error bars represent standard error.

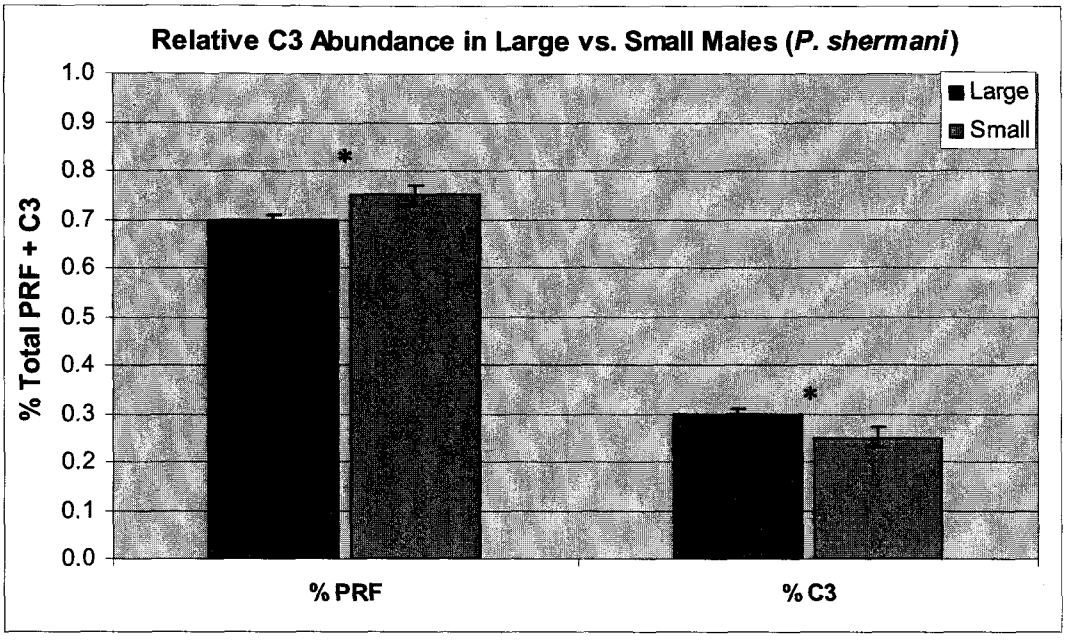

Figure 31. The relative proportion of non-PRF protein C3 in relation to the total volume of PRF. While 85% of the pheromone mixture is composed of PRF (22 kDa) and PMF (7 kDa), the next most abundant protein is by far C3 (18 kDa). Proportions were calculated (for each individual) by taking the total integrated area of all PRF isoforms, adding it to the area of C3, and dividing the area of each component by the total. Essentially this provides the PRF:C3 ratio. There is a significant difference in C3 proportion between Large and Small individuals, with larger individuals having a higher ratio of C3:PRF (two-tailed T-test; p=0.04).

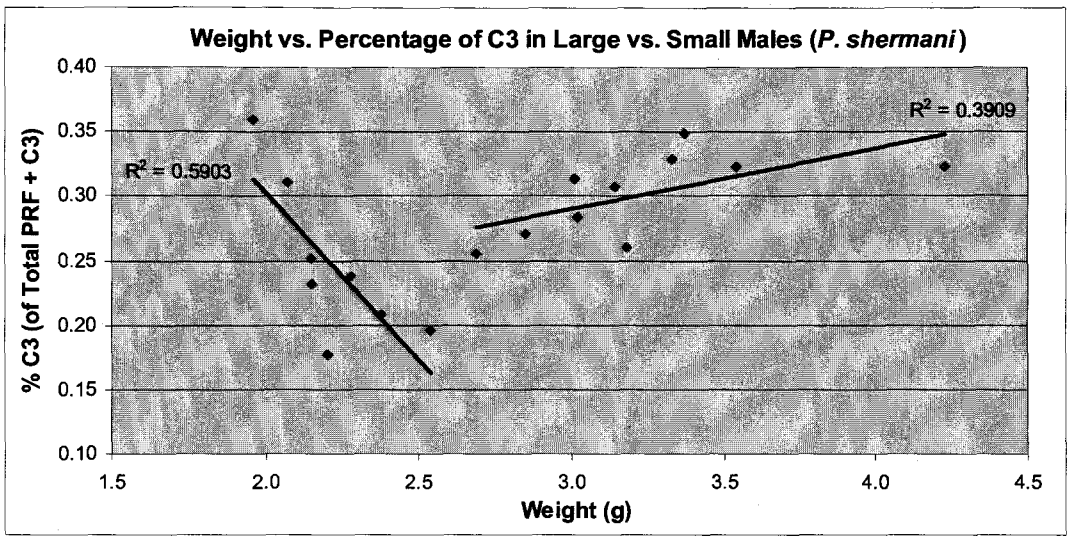

Figure 32. The percentage of protein C3 (in proportion to PRF) as compared to the weight of the source-male. Large males are depicted as red dots, while Small males are depicted as blue dots (as they relate to the treatment groups of Figure 30 and 31). Males were originally sorted into size-groups visually, and no experimental manipulation was done. Thus, the weight of the source-animals serves as a good basis for a correlation with relative component proportions. When analyzed as groups, there was a significant difference in C3 proportion between groups (Figure 31), but when analyzed as a continuous data series, there is a curious trend in which the smallest individuals began to resemble the larger animals in terms of C3 proportion (see Discussion).

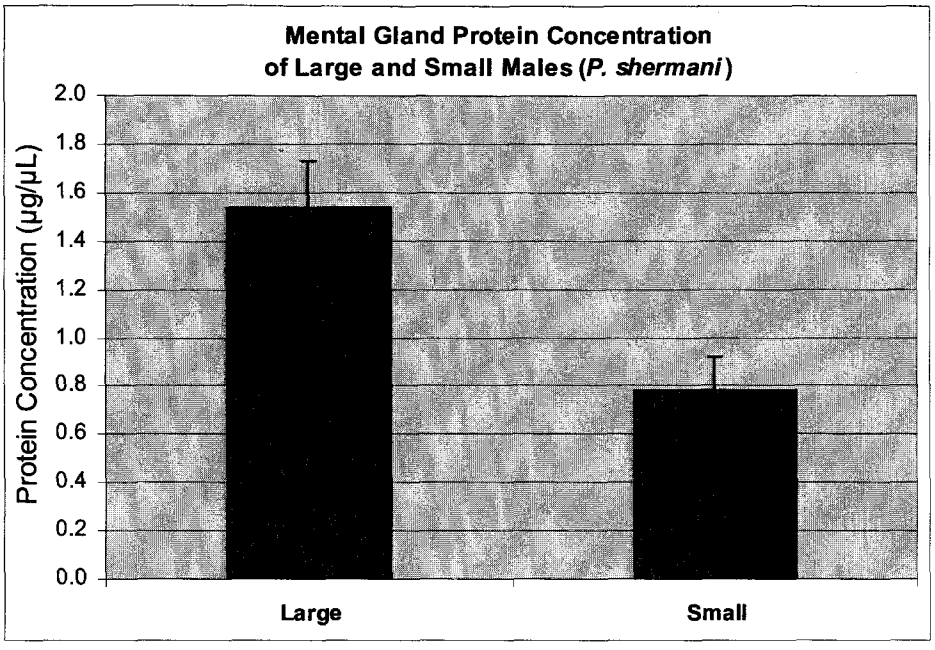

Figure 33. Average protein concentrations of Mental Gland extracts from Large and Small male Red-legged salamanders. Large males had a significantly greater amount of secretory protein than Small males (two-tailed T-test; p=0.006). Error bars represent Standard Error.

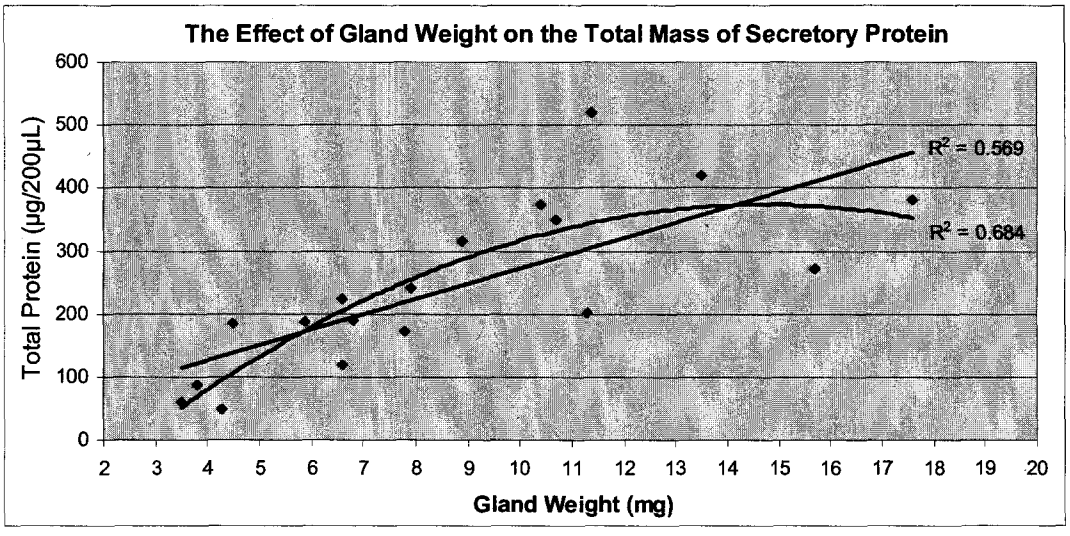

Figure 34. The total amount of secretory protein as a function of the weight of the Mental Gland of the source-animal. There seems to be a proportional relationship in which larger glands are capable of secreting more protein (as would be expected). Interestingly, this seems to be true only to a point, at which the curve begins to level off. Two regression lines were included (linear and curvilinear) to demonstrate the best fit of the curvilinear line.

Discussion

The most evident difference in pheromone signaling between male *P. shermani* of different sizes consisted of the total amount of protein secreted (Figure 33). This is analogous to the findings of Section II.C (see Figures 20-24) in which High-Quality male *P. cinereus* had substantially more protein yield in both the MG and PCG. In such a complex communication system where the pheromone profiles are so highly variable, one thing has been shown to be perfectly clear: more energy gained will allow for a greater amount of pheromone production. This is likely to be the initial mechanism with which both female mate-choice and territory-defense are facilitated (as discussed fully in Section II.C).

The findings of this experiment (in conjunction with those from Section II.C) conclude that the amount of protein produced and released by the sender is responsible for a large proportion of the individual variation in pheromone communication. However, this does not mean that changes in protein ratios aren't also of great importance to the physiological and behavioral effects on the receiver. It should be kept in mind that essentially every peak and/or band of the biochemical analyses performed throughout this research, is a molecule that is actively secreted by the source glands: these analyses are performed on the AchCl extract solution (not from homogenized cells), and thus only secretory products are obtained.* This is addressed in this manner because every one of these molecules is produced to carry out a specific physiological function. Just as the amount of total protein present will elicit stronger or weaker responses accordingly, the amount of each one

* With the exception of trace amounts of intracellular by-products from surgical removal.

of the component proteins will dictate the degree to which it is capable of carrying out its necessary function. When considering the number of components and all of their possible permutations, changing component ratios produce a huge degree of information capacity (in the spirit of Wilson, 1975; Chapter 8).

In this particular experiment, the ratios of PRF components could not be definitively associated with males of differing sizes. None of the PRF isoforms significantly differed between male size-groups (Figure 30). The closest candidate was isoform C2, which demonstrated a trend of greater abundance in larger individuals. The main problem with the experimental design was a low sample size, and the inability to create rigorously-matched size groups that differed by a substantial amount. These animals were collected from Macon County (NC), by myself and members of the Houck and Feldhoff research groups; however, the number of animals were a limiting factor because of the number of other studies being carried out by these researchers. Thus, size-groups had to be arranged from a small number of animals, and the result was more of a gradient than distinct size-groups (see Figure 32). Therefore, when attempting to analyze the PRF ratios between the two groups, the degree of intermediate individuals could very well have diluted the findings.

In contrast, it could very well be that PRF ratios are completely independent of the size of the sender. Indeed, previous research has revealed a tremendous amount of variability in the presence/absence and proportion of PRF isoforms between individuals (see Appendix E for isoform ratios of individual males) and between populations (Rollman et al., 2000). It may be that the regulation of PRF

expression is more dependent on evolutionary genetics than it is on environmental conditions. Rollman et al. (2000) did identify some significant differences in isoform abundance between different geographic locations, but the results were still relatively ambiguous. Even considering the results of Rollman et al., (2000), there do not seem to be any perfectly clear trends between geographic locations, because the presence/absence of isoforms between certain individuals within the same geographic population is so incredibly varied. Future research is being developed to investigate the differences in neural response to PRF isoforms, in order to more clearly understand their effect on the receiver and hopefully infer additional information about their function and regulation. Research into the specific factors controlling the genetic regulation of PRF isoforms is also needed.

While specific PRF isoforms did not vary according to the two size-groups, the non-PRF protein C3 did exist in a significantly greater proportion in large males (C3:PRF ratio; Figure 31). This is an exciting finding because practically nothing is known about C3 function or structure (other than its size, charge and relative hydrophobicity; Feldhoff et al., unpublished data). As stated above, the size distribution between groups was really more of a gradient than well-controlled, distinct size differences. When analyzing the proportion of C3 over the entire size-range (Figure 32), there was an interesting trend in which the percentage of C3 increased with weight in the largest males, but there was an inversion of this trend for the extreme end of the lower-weight animals. Thus, there was a U-shape trend in which the largest and smallest males contained the highest proportion of C3. If the indication of this experiment is correct (that C3 does convey information coupled to

the size of the sender; i.e. Figure 31), then this could potentially be explained by an element of alternative male mating strategies. It could be that males at the furthest end of the size-range devote more of their energy into resembling the signal of a larger animal. This could be considered a situation of dishonest signaling, which has been thought to occur in other systems (Bee et al., 2000; Sadd et al., 2006). Quite frankly, too little is known about the function of C3 to draw any serious conclusions, and this could in theory be an anomaly. Additionally, the proportion of C3 was calculated as compared to the total of PRF; thus, the smallest animals in question could simply have been producing less PRF, thereby indirectly increasing the proportion of C3. In analyzing the hard data, this does not prove to be the case.* Needless to say, further experimentation with more rigorous size-matching and a more quantitative analysis of isoform abundance is needed.

In considering alternative strategies and individual variation, the size of the MG itself must be addressed. Not only did larger animals produce a higher total protein yield (Figure 33), but this trend was also observed when related back to the size of the actual Mental Gland from which it was secreted (Figure 34). As would be expected, there was a clear trend of larger glands producing larger amounts of protein (although the effect seemed to level off at the higher end of the range). Interestingly, the size of the gland is not always immediately proportional to the size of the animal itself. There is of course a higher likelihood that a large male will

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* Actually, the "small" males had a slightly larger total PRF absorbance ($\overline{X}$ = 2843086.9) than "large" males ($\overline{X}$ = 2699840.9), though this was not statistically significant in either direction (two-tailed T-test; p=0.42).

possess a larger MG, but it is not always the case. Similarly, there are occasionally smaller individuals with surprisingly massive Mental Glands (see Appendix F for data). As this gland is hypertrophied every year prior to mating (Sever, 1976b), and as the production of this gland is no doubt related to the amount of energy obtained by the individual that year, it could be that the size of the MG itself is the mechanism by which information of foraging-ability/energy-acclimation could be indirectly conveyed to females (through the ability to ultimately produce more protein).

Overall, this experiment provides the first evidence* that the proportion of a pheromone component is affected by the size of the sender (Figure 31), but the data is still correlational. I am currently developing new extraction methodology in order to conduct a paired statistical analysis, in which the pheromone profiles of an individual can be compared to the same individual under different experimental conditions. This will provide tremendous insight regarding the degree to which pheromone profiles of an individual change over time. This will also enable an entirely new method for investigating the fluctuations of certain pheromone components as they correspond to specific environmental conditions, which will begin the process of identifying which pheromones are responsible for conveying which aspects of information.

* This is certainly true for Plethodontids and other caudate amphibians, as well as the remaining literature to the best of my knowledge (although there may be examples in other systems of which I am not aware).

II.E. POSTCLOACAL GLAND HISTOLOGY

Objective

The purpose of this section is to verify that the region of tissue taken from the Postcloacal area for previous biochemical analysis did contain the specific types of granular glands that have been previously characterized as the site of scent-mark productions. Simons et al. (1992; 1994; 1999) have established the Postcloacal Gland as the area of the body responsible for producing territorial advertisement (scent-marks). This can be visually observed through the "Postcloacal Press" behavior, in which the salamander presses the ventral length of its tail firmly to the substrate. Scent-marks can also be seen in artificial territories in a laboratory setting. As previously described, these advertisements are capable of conveying a great deal of information, and this was the broad focus of this research. However, in order for the biochemical analyses completed to provide reliable insight, it was necessary to verify that the tissue taken did in fact contain the integumentary glands that are thought to be the source of the scent marks.

Various glandular regions of *P. cinereus* have been previously described through histological methods by Hecker et al. (2003). This study revealed that the Postcloacal tissue contains a dense arrangement of "S1" granular (or serous) glands, which differ in their morphology and staining pattern than "S2" granular glands, found to be highly prevalent in skin from the Dorsal Tail Base. The Dorsal Tail Base is utilized in courtship rituals, while the Postcloacal Gland (or Ventral Tail Base) is utilized for scent-marking. The differences in function are obviously related to the different histochemical characteristics observed. S1 granular glands stain strongly

for Periodic Acid Schiff (PAS), and thus indicate a neutral carbohydrate composition. S1 glands also stain mildly for Indocyanine, because of a proteinaceous component. S2 glands do not stain positively for PAS, but stain more strongly for Indocyanine; there is less carbohydrate and more protein present. In both cases, the granular glands stain strongly (pink) for Eosin. The differences between S1 and S2 granular glands were not of particular interest to this study, because of their differentiation based on location. It was, however, necessary to distinguish that granular glands were present at all in the tissue samples taken, and that the glands in question were not simply Mucous glands (another abundant amphibian integumentary gland).

Mucous glands stain negatively for Eosin, and are thus easily distinguished from granular glands through the most basic histochemical method (H+E staining). Mucous glands of the integument are also substantially smaller than the S1 and S2 granular glands. In order to verify the presence of S1 granular glands, Postcloacal tissue was surgically removed and analyzed histologically. It was predicted that the large acinar granular glands would be present, as distinguished by strongly staining eosinophilic secretory granules in the lumen. It was also predicted that the S1 granular glands would be clearly distinguished from the smaller, non-eosinophilic Mucous glands, which would also be present throughout the tissue samples.

Methods

Male salamanders were collected from College Woods in Durham, NH in October, 2009 (IACUC #080502). Animals were housed in the lab at 20°C on a 14:10 L:D cycle, and fed a diet of *Drosophila* and *Enchytraeus* ad libitum. Four salamanders (2 male, 2 female) were selected randomly for surgical removal of the Postcloacal tissue. Prior to surgery, animals were anesthetized in 7% ether for 10 minutes. Surgical removal of the glands was identical to the protocol in Section II.B. Upon removal, glands were immediately placed into 10% Neutral Buffered Formalin for 48 hours in order for chemical fixation. After the fixation period, all glands were processed through standard histological processing methods (Carson, 2009) in preparation for light microscopy.

All sections were taken on a rotary microtome, at 5 μm intervals. Sections were taken in the coronal plane, for the full depth of the tissue sample (horizontally down through the sample of skin; as opposed to a transverse section through the tail). Sections were placed onto slides and air-dried for 24 hours. Slides were then stained through standard Hematoxylin and Eosin (H+E) staining methods (Carson, 2009). Slides were analyzed via light microscopy, and digital images were taken with an Olympus DP-20 digital camera.

Results

Histological analysis revealed clear demonstration of S1 granular glands in the Postcloacal tissue of male and female *P. cinereus* (Figure 35). Granular glands could be distinguished by their acinar structure, with secretory cells surrounding a large circular lumen. The glands could also be identified by their strong affinity for Eosin, with light pink cytoplasm in the secretory cells, but also dark pink secretory granules in the lumen of the glands (Figure 36 and 37). The S1 granular glands are easily distinguished from Mucous glands by the lack of eosin staining and much smaller size in the latter (Figure 35; 36).

The granular glands extended into the stratum compactum of the dermis, until they were finally met by a basement membrane overlying skeletal muscle of the tail. Mucous glands also seemed to be at shallower depths of the dermis than granular glands, and thus appear mostly at the periphery of the samples (i.e. Figure 35). This is because the tissue being sectioned was curved as a function of both the surgical removal, but also the natural shape of the tail from which the tissue came. Therefore multiple depths of the tissue were being cut in an individual section, with the shallower depths toward the periphery.

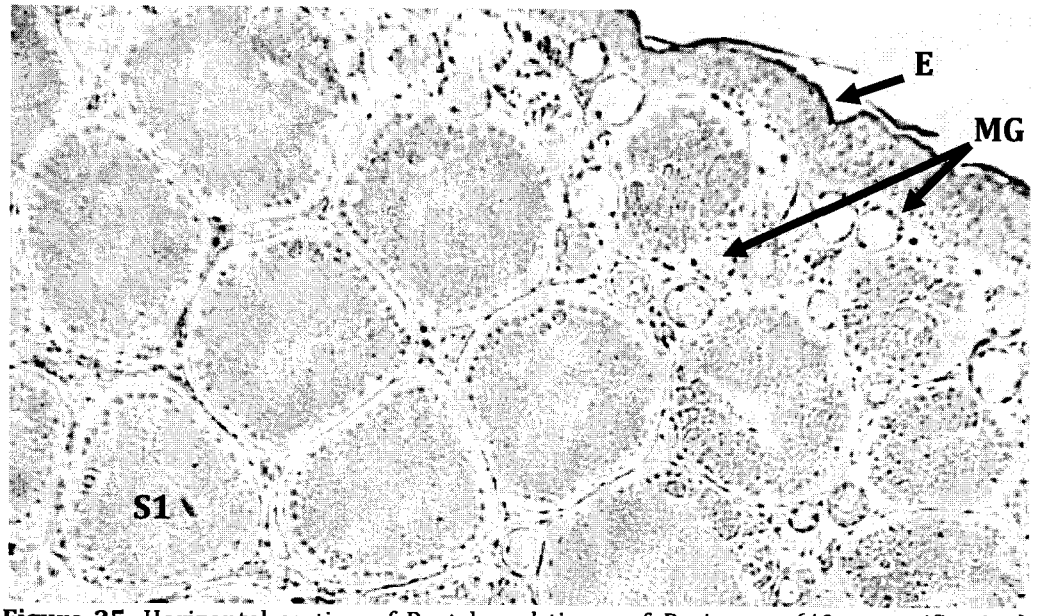

Figure 35. Horizontal section of Postcloacal tissue of *P. cinereus* (40x magnification). Large S1 granular glands can be seen throughout the tissue (S1), adjacent to numerous smaller mucous glands (MG). Epidermal tissue can also be seen at the periphery (E).

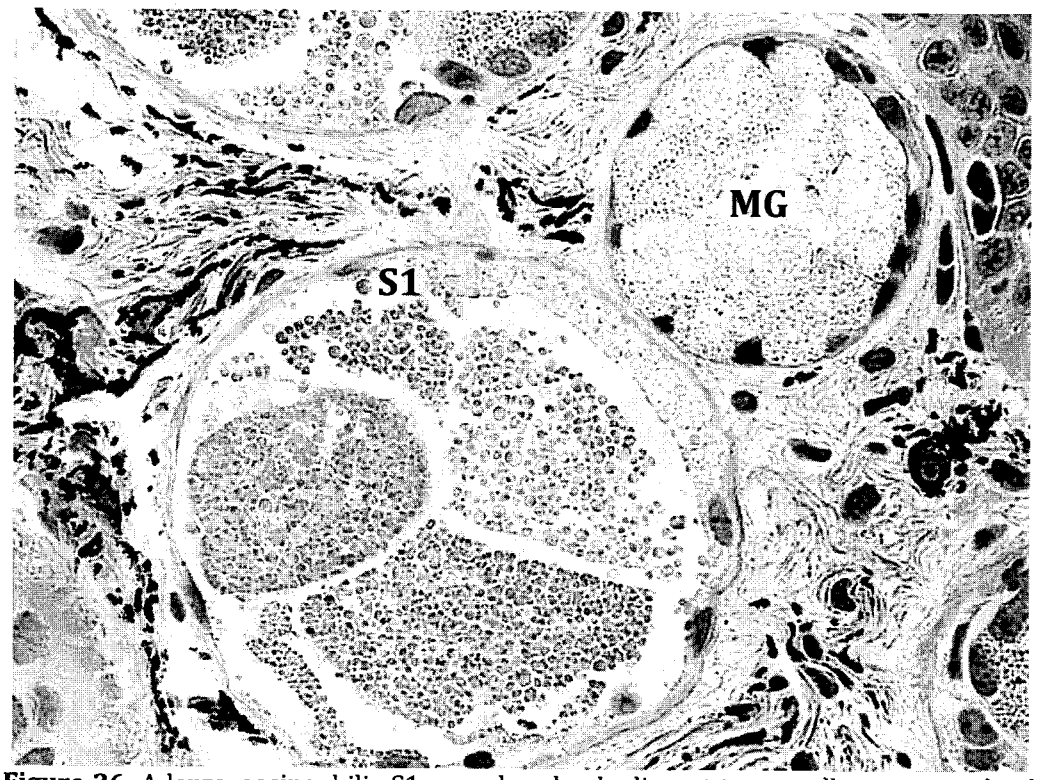

Figure 36. A large, eosinophilic S1 granular gland adjacent to a smaller mucous gland (MG). The difference in size and staining characteristics allow for these glands to be easily distinguished. Melanin pigments can also be seen deposited throughout the tissue (black areas). Magnification = 400x.

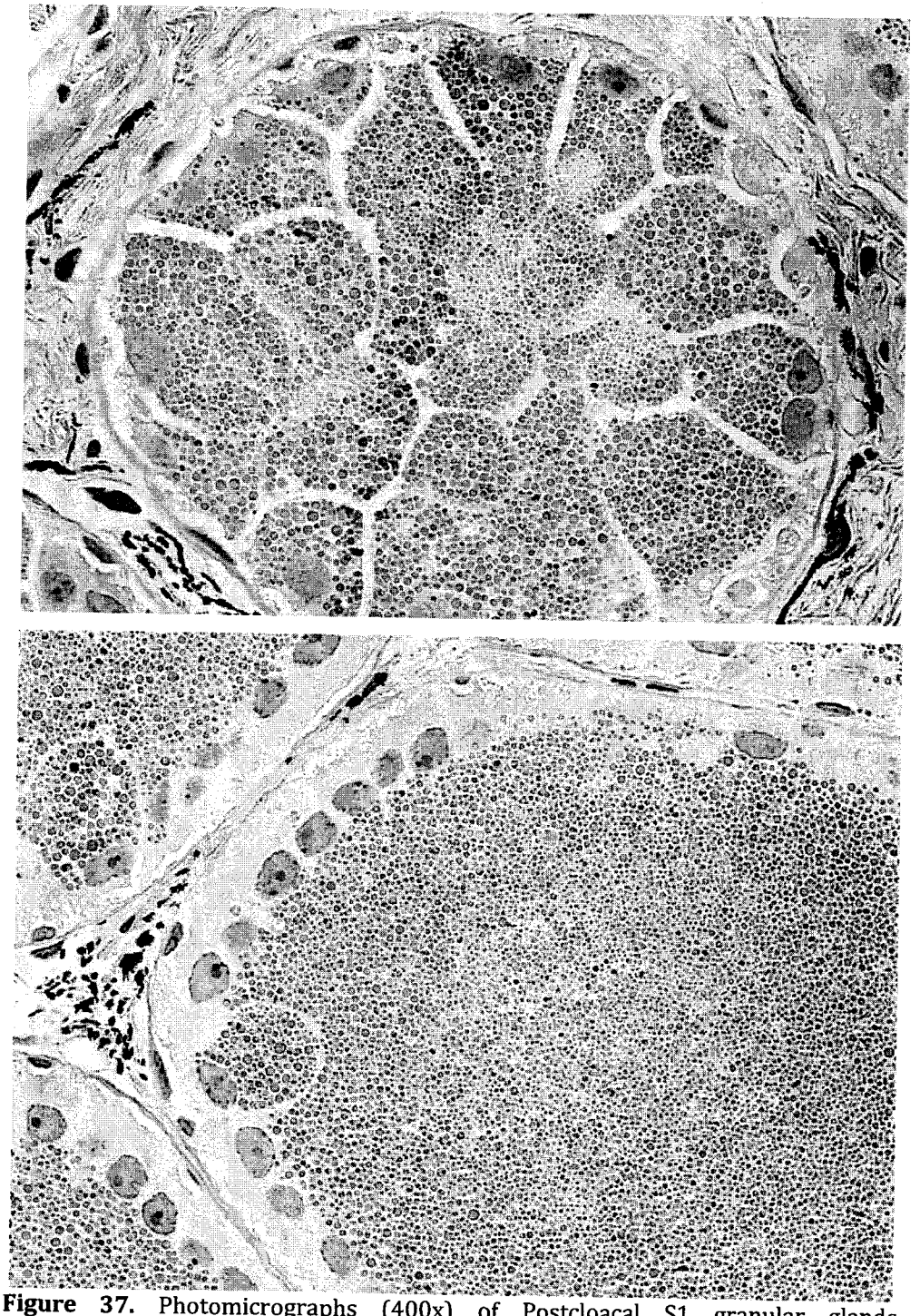

Figure 37. Photomicrographs (400x) of Postcloacal S1 granular glands demonstrating the secretory granules, as well as glands in various stages of replenishing the secretory product. The large, bright pink areas with numerous small circles is the lumen of the granular gland, while the cells lining the lumen stain less strongly with Eosin; the nuclei can clearly be seen and stain strongly with Hematoxylin. Melanin can also be seen, as well as a (nucleated) erythrocyte.

Discussion

The histological analysis of the ventral caudal integument of *P. cinereus* revealed distinct S1 serous (granular) glands (*sensu* Hecker et al., 2003). The granular glands in question also clearly resemble the descriptions and microscopy from past histological analysis of the integument from various glandular areas (*P. cinereus* – Simons and Felgenhauer, 1992; Simons et al., 1999; Hecker et al., 2003; *P. shermani* – Largen and Woodley, 2008; *Notopthalmus viridescens* – Pool and Dent, 1977; Pool et al., 1977; *Ensatina eschscholtzii* – Fontana et al., 2006).

Other expected glands could also be observed, and these were again identified based on the descriptions and microscopy from the above citations. The major non-granular gland components were mucous glands (Figure 35/36). Mucous glands were easily distinguished from granular glands because of the lack of strong staining with Eosin and their much smaller size (Hecker et al., 2003). In contrast, the secretory products of the serous lumen stain strongly with Eosin (Figure 36), and it was mainly for this reason that the use of the standard H & E staining was considered sufficient for the analysis. The objective of this largely control-oriented study was to definitively determine that granular glands were present in the tissue samples after the standard surgical removal procedure, and the histological analysis revealed that this was the case. Thus, it can be positively concluded that the biochemical analyses of PCG extract (Section II.B.2) were from S1 serous glands.

III. CONCLUSIONS AND IMPLICATIONS

The main objective of this research was to further elaborate upon the capabilities of the Plethodontid chemical signaling system in question, and explore the biochemistry through which variable series of information can be conveyed. In doing so, aspects of the total quantity of the signal, relative component ratios, and specific pheromone isoform ratios were investigated. The role of each of these biochemical variations will be synthesized further below, but first the conclusions from the behavioral analyses completed should also be addressed.

A great deal of previous behavioral analysis has revealed the impressive amount of information that can be inferred from pheromone signals deposited on the substrate (Section I.A.4). The behavioral function of particular interest in the current research has been the phenomenon of female mate-choice. This behavior was chosen because there is a strong selective pressure in place for females to preferentially associate with the scents of males with whom she will garner the greatest reproductive fitness. It is clear that pheromones play a large role in facilitating mate-assessment and courtship in Plethodontids (Section I.A.2 and I.A.4): pheromones provide seemingly honest signals of mate-quality through territorial advertisements, but also aid in convincing females to commence and complete the courtship ritual. In making this choice, females should be strongly selected to mate with larger and older males (Houck, 1988; Mathis, 1991; Marco et al., 1998; Verrell, 1995) with low parasite-loads (Maksimowich and Mathis, 2001) and a positive energy-budget (Walls et al., 1989; Jaeger and Wise, 1991), as all of these factors will obviously affect female fitness. This behavior therefore provides a reliable measure

to asses which information can and cannot be inferred through a pheromone medium, because females will be strongly selected to make clear associative preferences between differing quality-signals. This has been shown to be the case in previous research, but also in the experimentation of this thesis.

While others have demonstrated the ability of females to preferentially associate with the scents of males on higher-quality diets (Walls et al., 1989; Jaeger and Wise, 1991), the source of these signals and the time-scale through which this information can be contained was still unknown. The research of the current thesis has shown that the information of diet-quality seems to be independent of cues from the fecal pellets themselves. This is not to say that there isn't necessarily other information that could be obtained by investigating fecal pellets as well, but this research has shown that actively-produced territorial scent-marks do in fact contain this information. In other words, it seems that males are actively conveying this information through pheromones, and it is not an epiphenomenon derived from investigating fecal cues. While the initial results demonstrating this phenomenon could not dissociate the factors of diet and size of the sender (Section II.A.2), further experimentation (Section II.A.3) revealed that females were able to differentiate between the scents of High- and Low-Quality diets over a very short time (one week after differential feeding).

This finding is profound in that it reveals what seems to be a surprisingly instantaneous input to pheromone production. This provides relatively current information regarding the nature of the relationship of the source animal and its environment. It could be seen how instantaneous information such as this would be

of benefit to the female, as current condition of a potential mate is perhaps more important than other genetic predispositions. The subtext of the previous mate-choice hypotheses (e.g. "good-genes" - Zahavi, 1975; "sexy sons" - Weatherhead and Robertson, 1979) postulate the situation as if the genes of the current male are set in stone from birth, acquired from previously desirable fathers; simply possessing these desirable genes will prove the noble lineage of the current male and make him more desirable. This type of reasoning downplays the phenomenon of developmental plasticity, and ignores the incredibly powerful relationship between a developing animal and its environment. Our current understanding of the interplay between genes and the environment should lead us past this initial approach, and consider more intently the interplay between genes, biochemistry, behavior and the environment. This is not to say that genetic inheritance does not also play a large role, as certainly acquired predispositions will be of tremendous importance. The idea, however, is to fuse our previous understanding of inheritance with the overwhelming field of environmental plasticity (e.g. West-Eberhard, 2003).

The next looming question of exactly how this phenomenon is conveyed through pheromones was then addressed. The first (and most conclusive) result elucidated the relationship between the energy-budget and the total amount of secretory protein of the sender (Section II.C). These findings provide clear evidence for the hypothesis that males on higher-quality diets would possess more pheromone molecules, and it seems that this is a definite mechanism for conveying quality. The production of pheromone molecules (which include proteins, carbohydrates and lipids) is obviously reliant upon the ability to obtain the

appropriate amount of energy required for their synthesis. This relationship no doubt exists as a directly proportional correlation, with more energy leading to more pheromone production. This provides a full gradient in which a given male's signal can fall, and females have been shown to be incredibly skilled in assessing the precise amount of protein present in a scent-mark (see discussion of female preference results from Section II.C; page 135). Thus, it seems that the volume of the entire pheromone mixture allows for a graded signal of energetic condition of the sender, and this is likely an incredibly important mechanism of mate-choice, as well as in the acquisition and defense of territories.

Signal strength may very well be the primary mechanism of mate-choice and territoriality, but there is also evidence for relative ratios being an important aspect of pheromone variability. These two concepts are not completely unrelated, as many of the pheromone molecules may simply be functioning on parallel pathways, and therefore the sheer amount of each component molecule will differentially affect the physiology and behavior of the receiver, but not as a function of their relative ratios *per se*. Essentially, the question still remains as to whether or not additional emergent aspects of information can be obtained from the component ratios themselves, and not simply the amount of each component separately. Quite frankly, this question still requires a great deal more experimentation, and permeates the broader field of chemical signaling and communication as a whole.

While further research is of course needed, this experimentation has revealed some important information about the relative component ratios of Plethodontid pheromones. For one, the degree of individual variability between

pheromone profiles is tremendous. This is true for both glandular regions of the integument tested, and also for both *Plethodon* species analyzed. Really, this can hardly be surprising based on the high degree of variability required to convey certain aspects, such as individual-identity. While not all of the pheromone analyses were incredibly definitive, all were still of great importance since none of them have previously been done in *P. cinereus*. These analyses will therefore provide the backdrop for a new avenue of future research. Even so, the biochemical analyses performed have allowed for some notable conclusions.

For one, it appears that the pheromone profiles of *P. cinereus* from NH and VA are surprisingly different. This may be essential in reconsidering species boundaries, which some researchers have already begun to question (e.g. Highton, 1999). Again, pheromones may be of tremendous importance in speciation, functioning as a premating isolation mechanism (Dawley, 1986a; 1986b). This research was also the first to investigate differences in the ratios of known pheromone isoforms as they relate to source-male characteristics. While the variability of PRF seemed to extend further than the size of the sender, the non-PRF component C3 was shown to be more abundant in larger males. This is important because literally nothing is known about the function of this molecule. This analysis certainly warrants future research, and provides the first candidate for the molecule that may be conveying size.

Overall, this research has shed new light on the pheromone composition of *P. cinereus*, as well as the relationship between the sender and its environment, and the role of pheromones in conveying this information.

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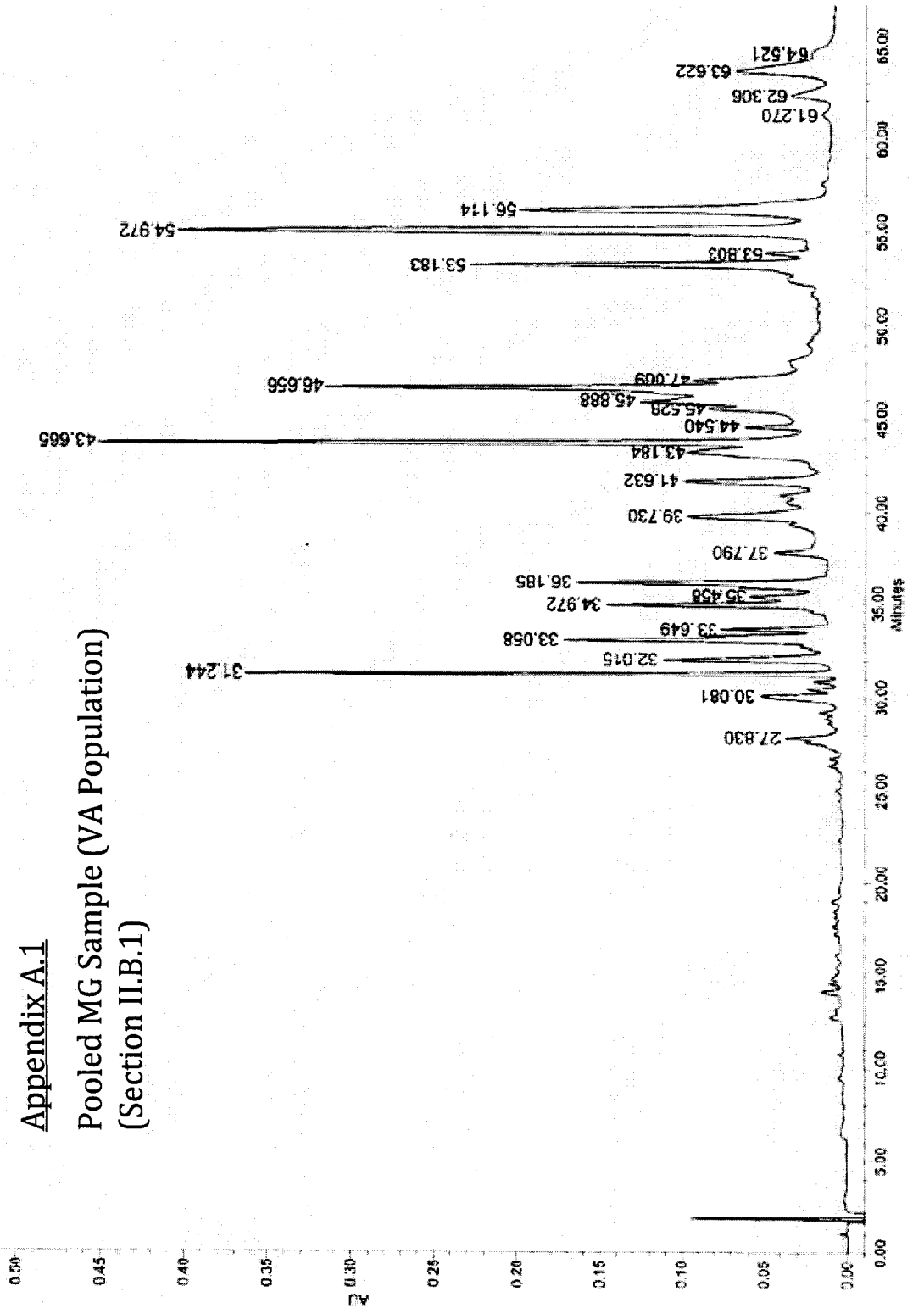
APPENDICES

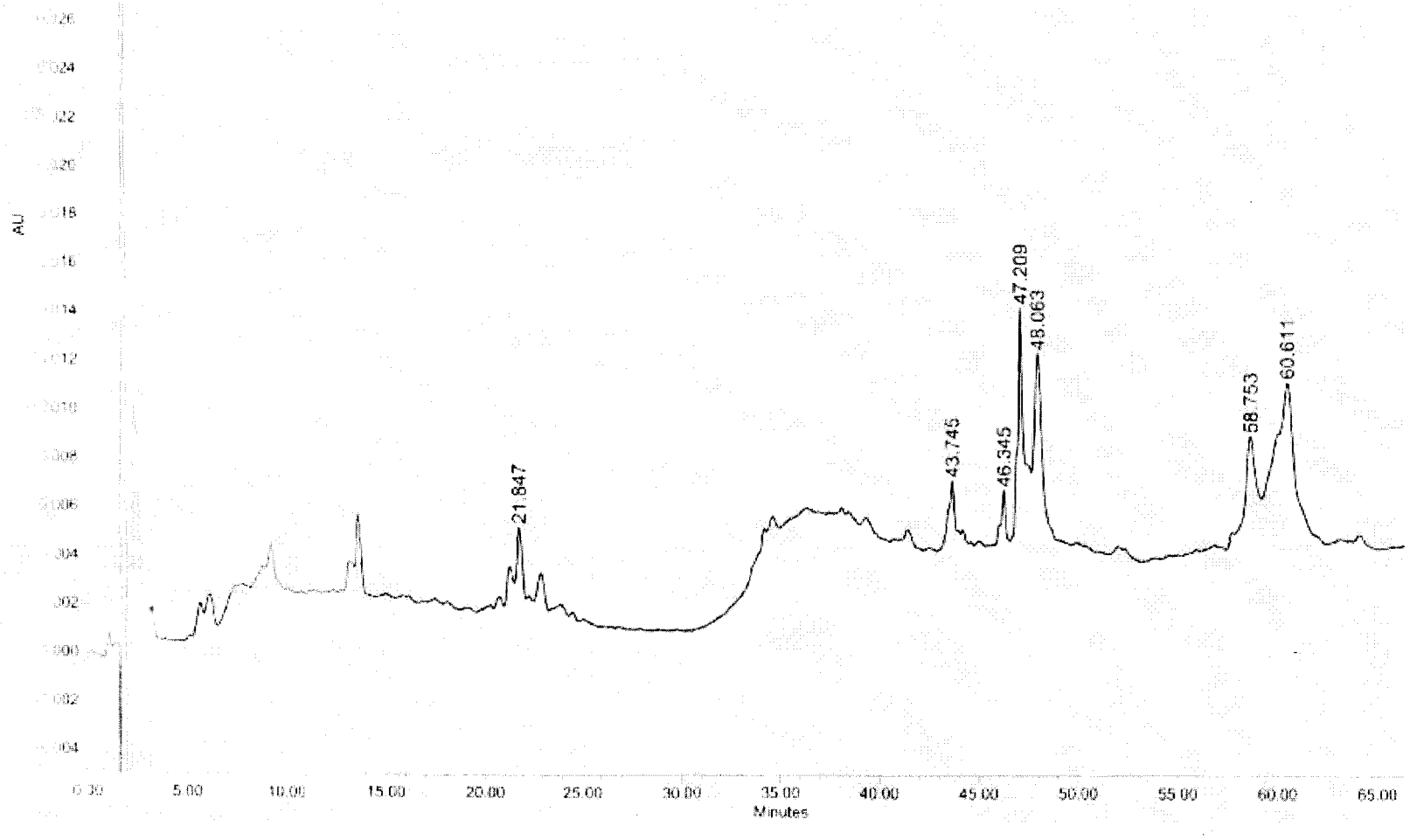
Appendix A.1

Pooled MG Sample (VA Population)
(Section II.B.1)

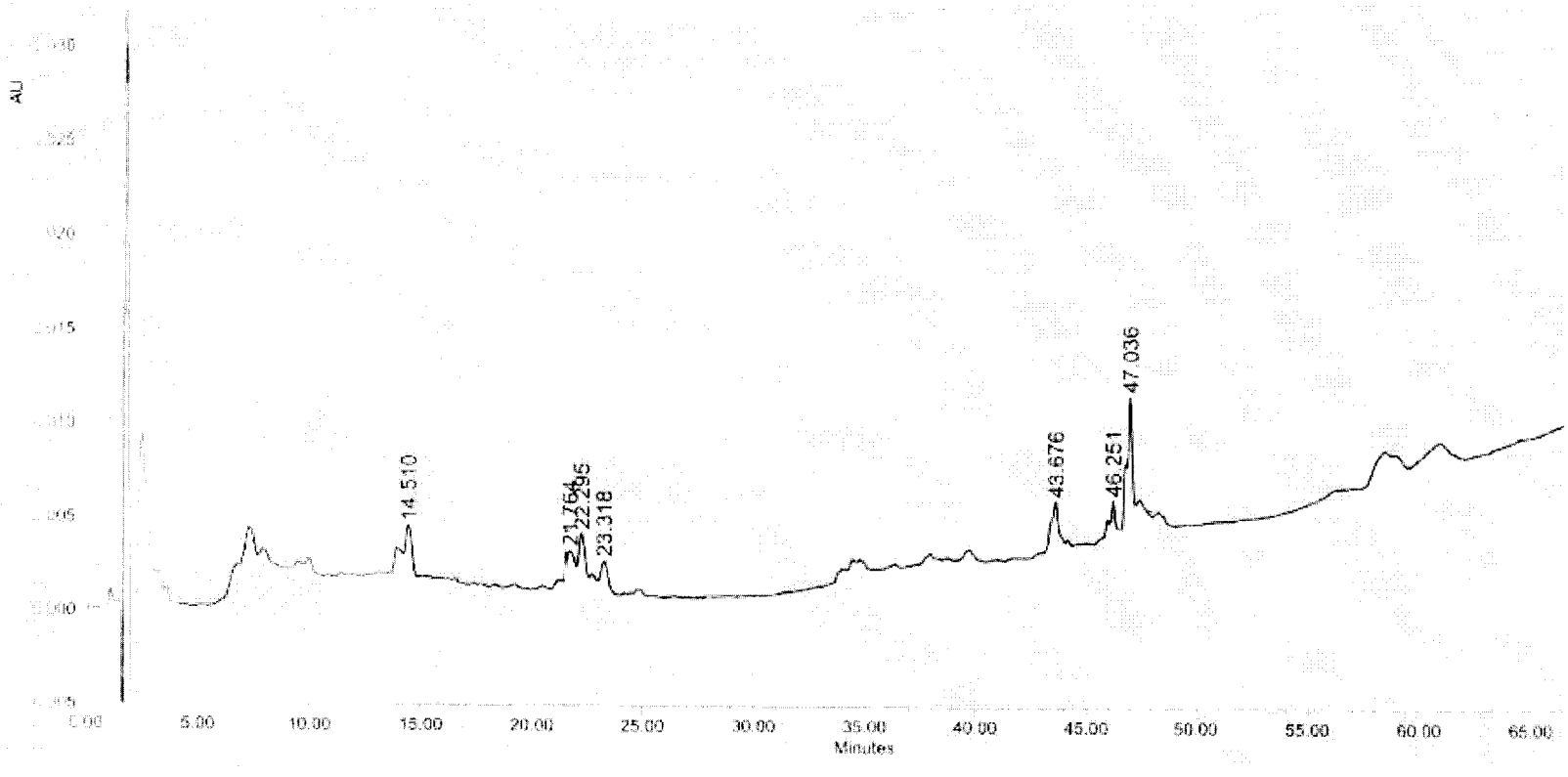


Appendix A.2

Pooled MG Sample (NH) – Run 1 (Section II.B.1)

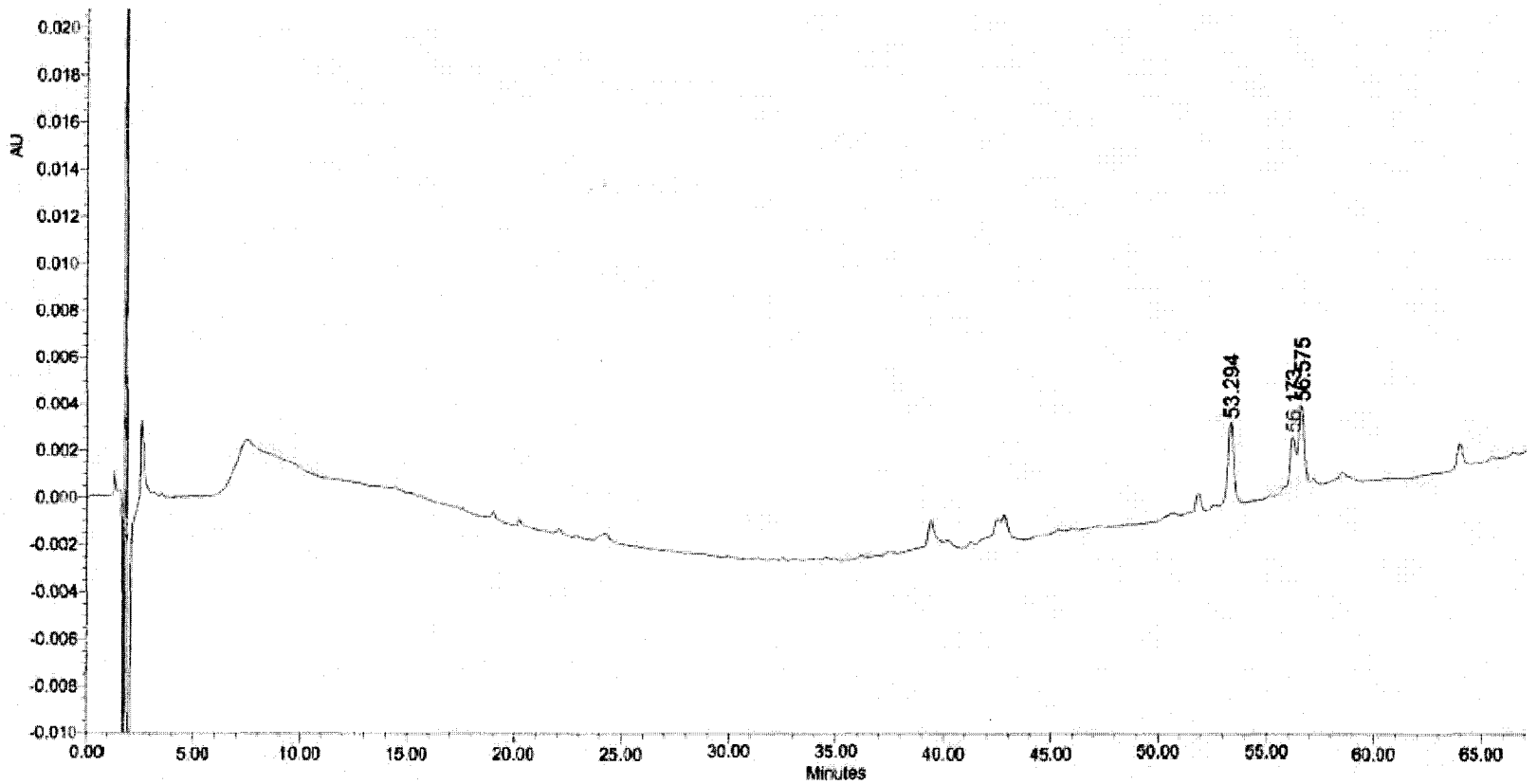


Pooled MG Sample – Run 2 (Section II.B.1)

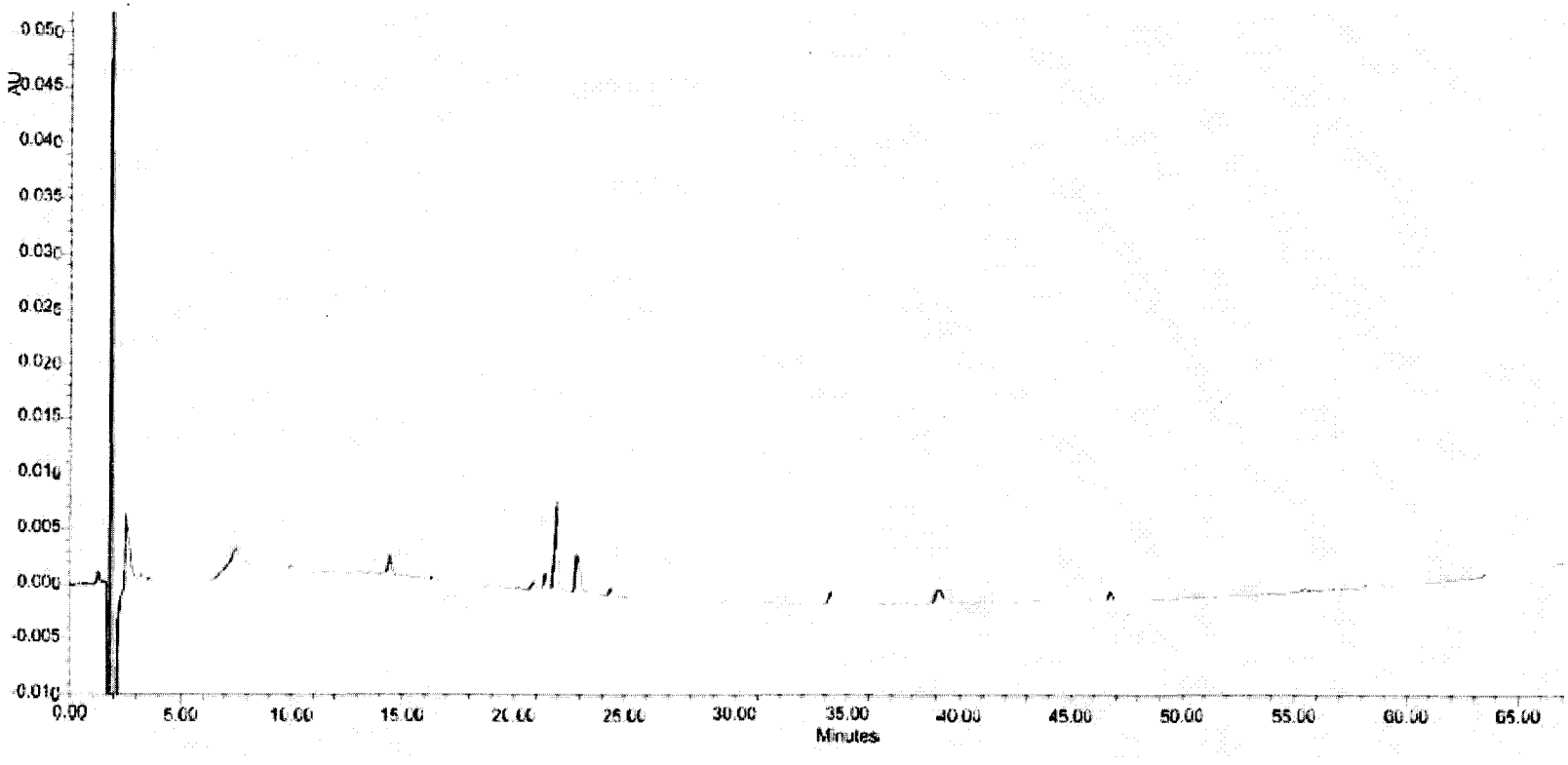


Appendix A.3

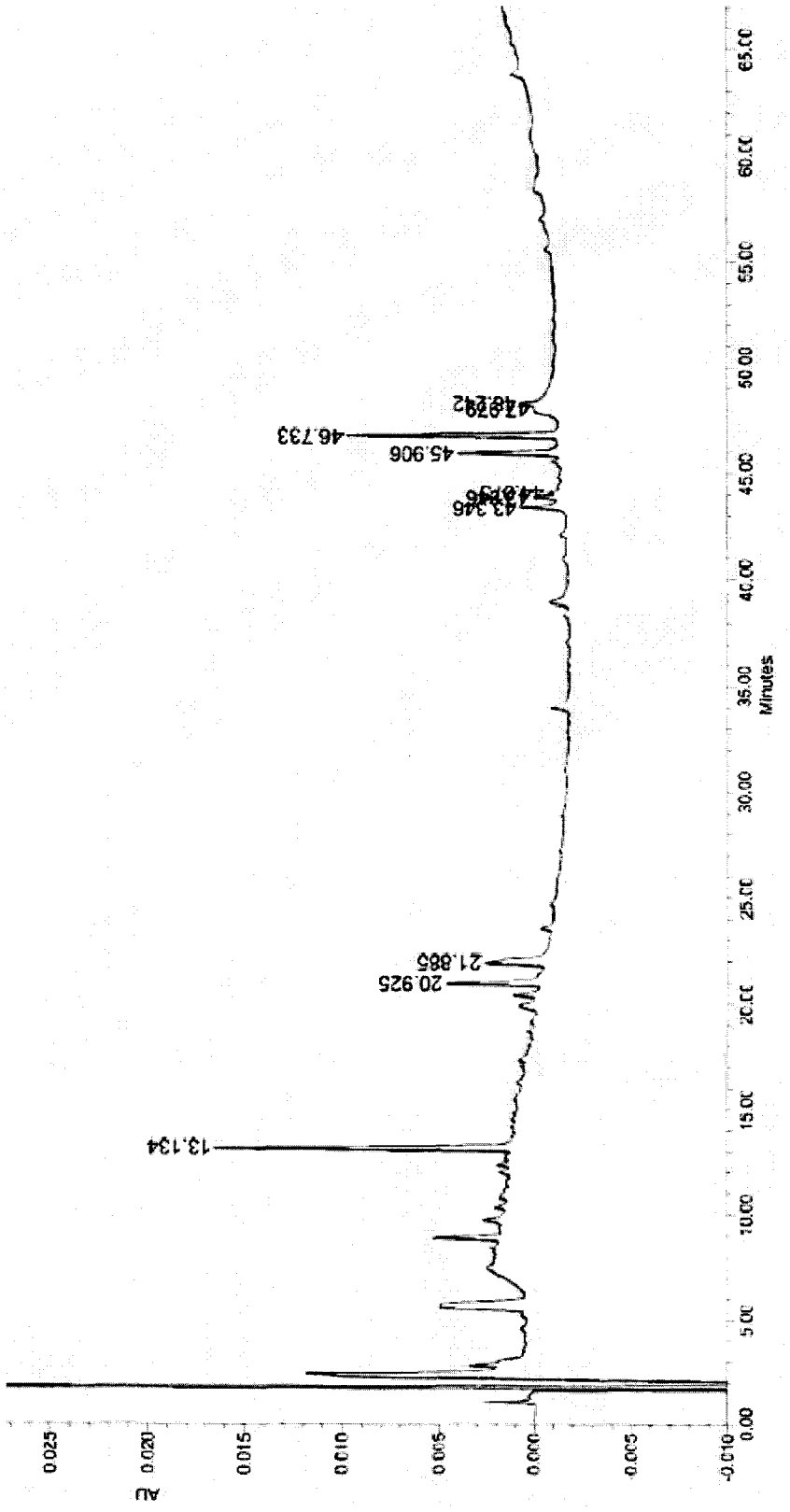
Individual MG Sample (Section II.B.1) – Male # 1



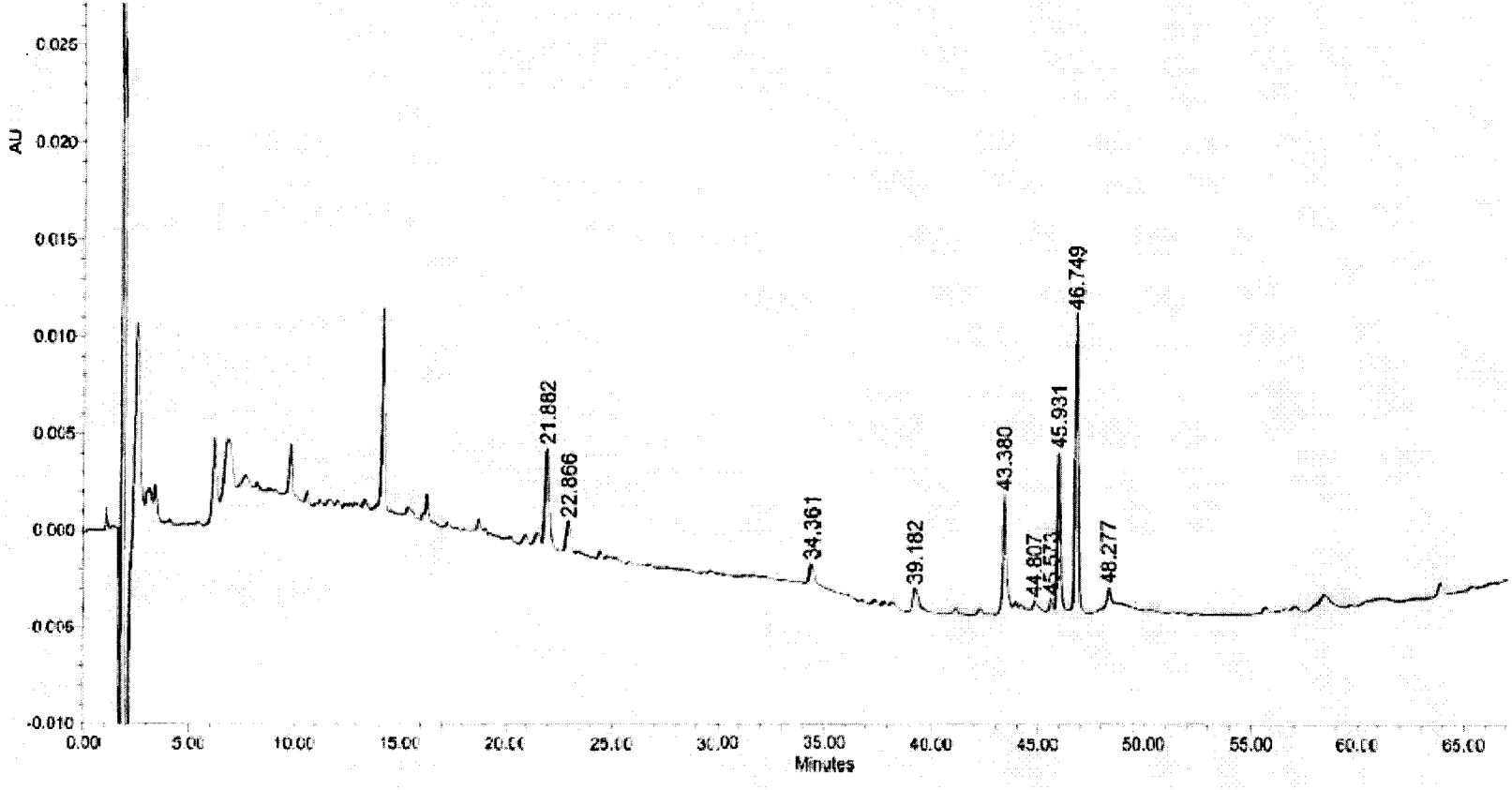
Individual MG Sample (Section II.B.1) – Male # 2



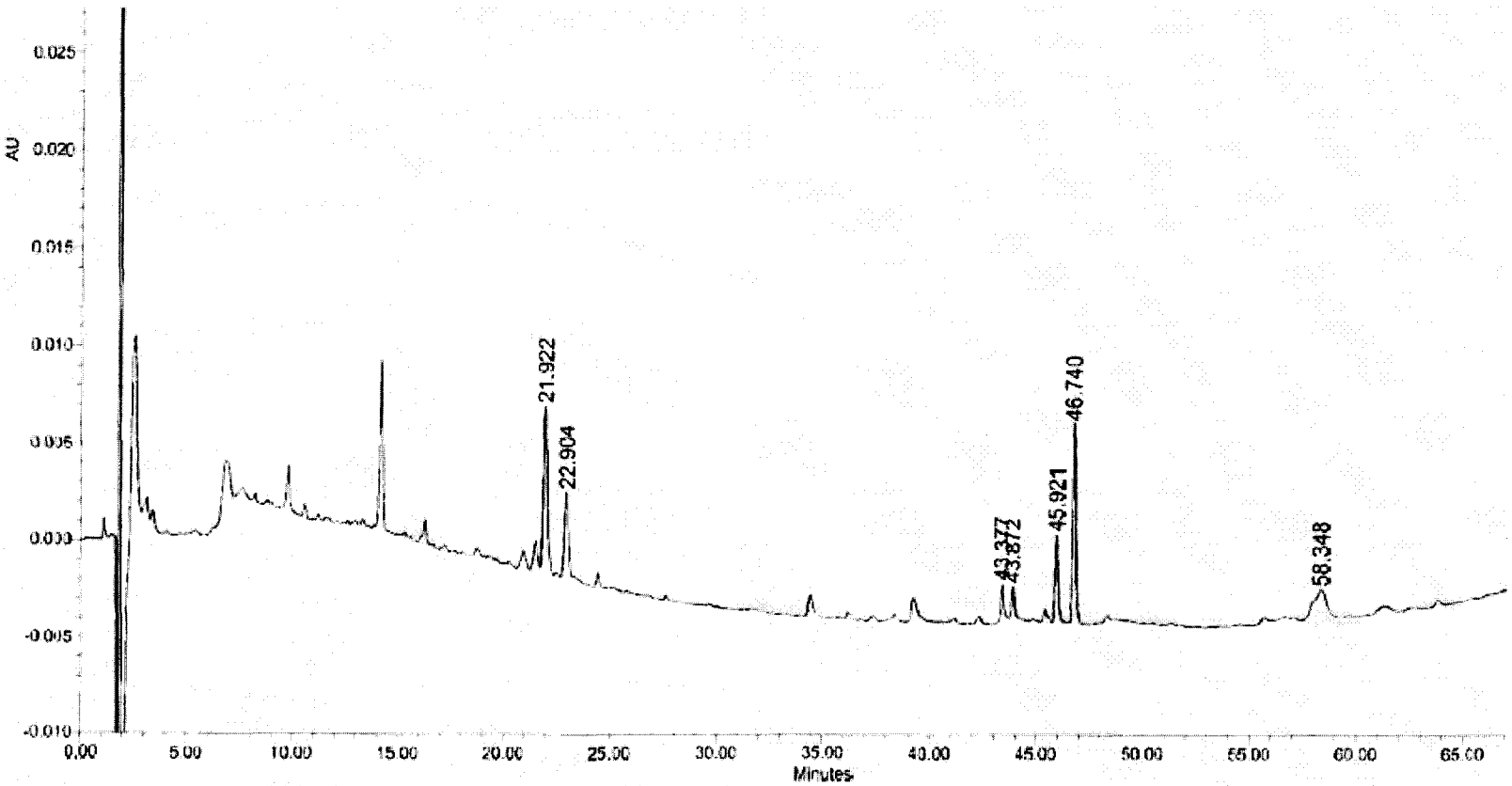
Individual MG Sample (Section II.B.1) – Male # 3



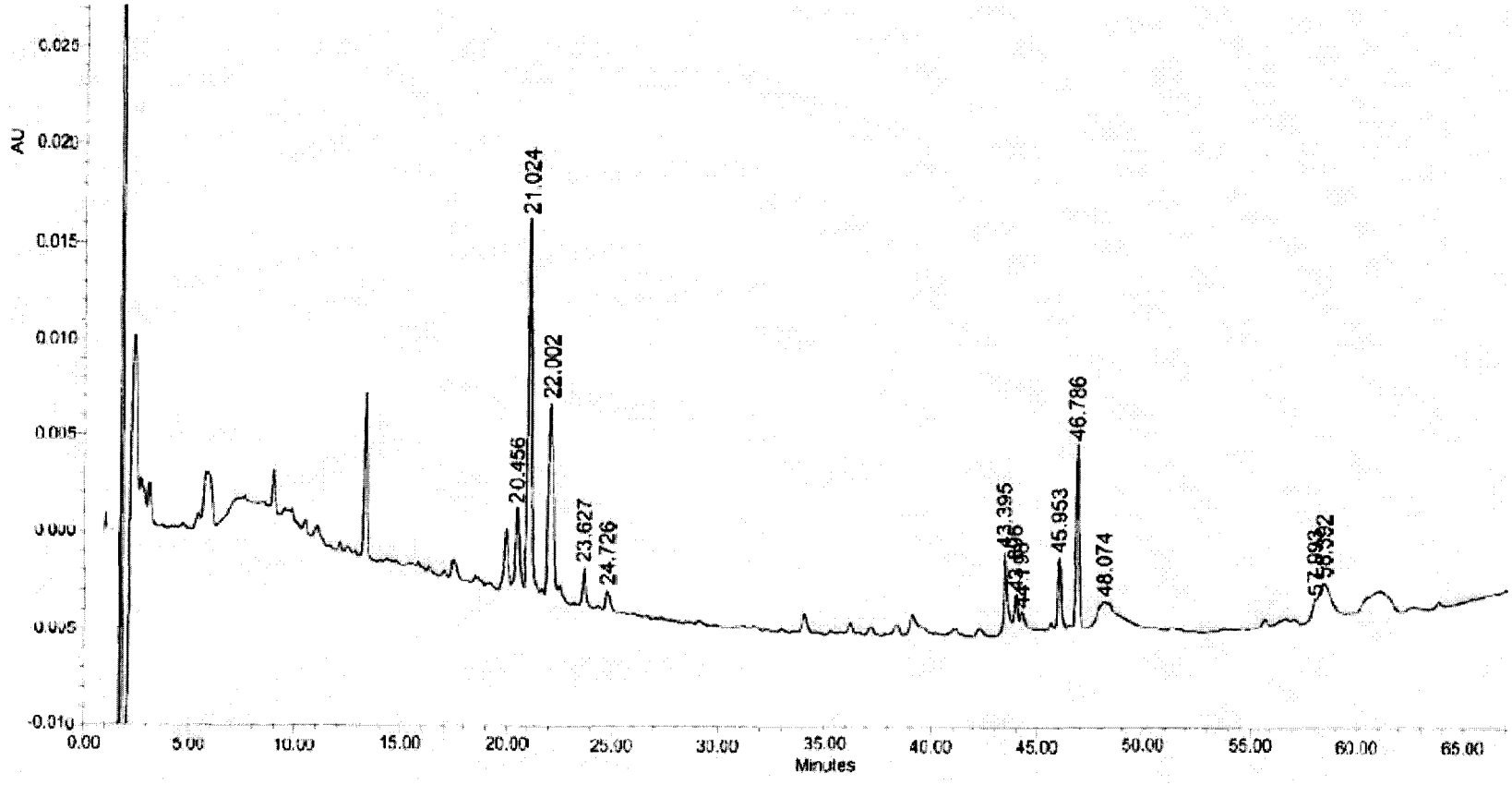
Individual MG Sample (Section II.B.1) – Male # 4



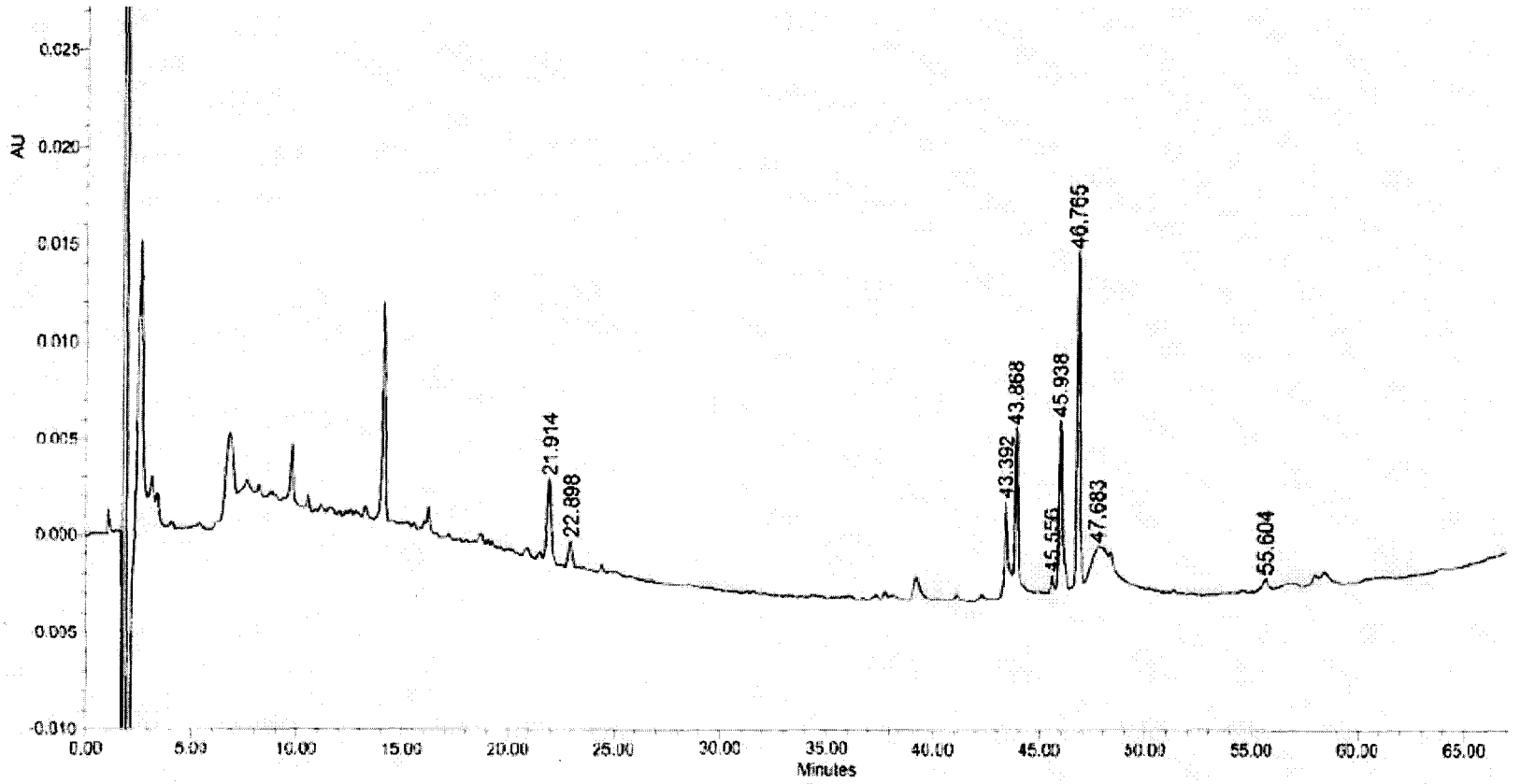
Individual MG Sample (Section II.B.1) – Male # 5



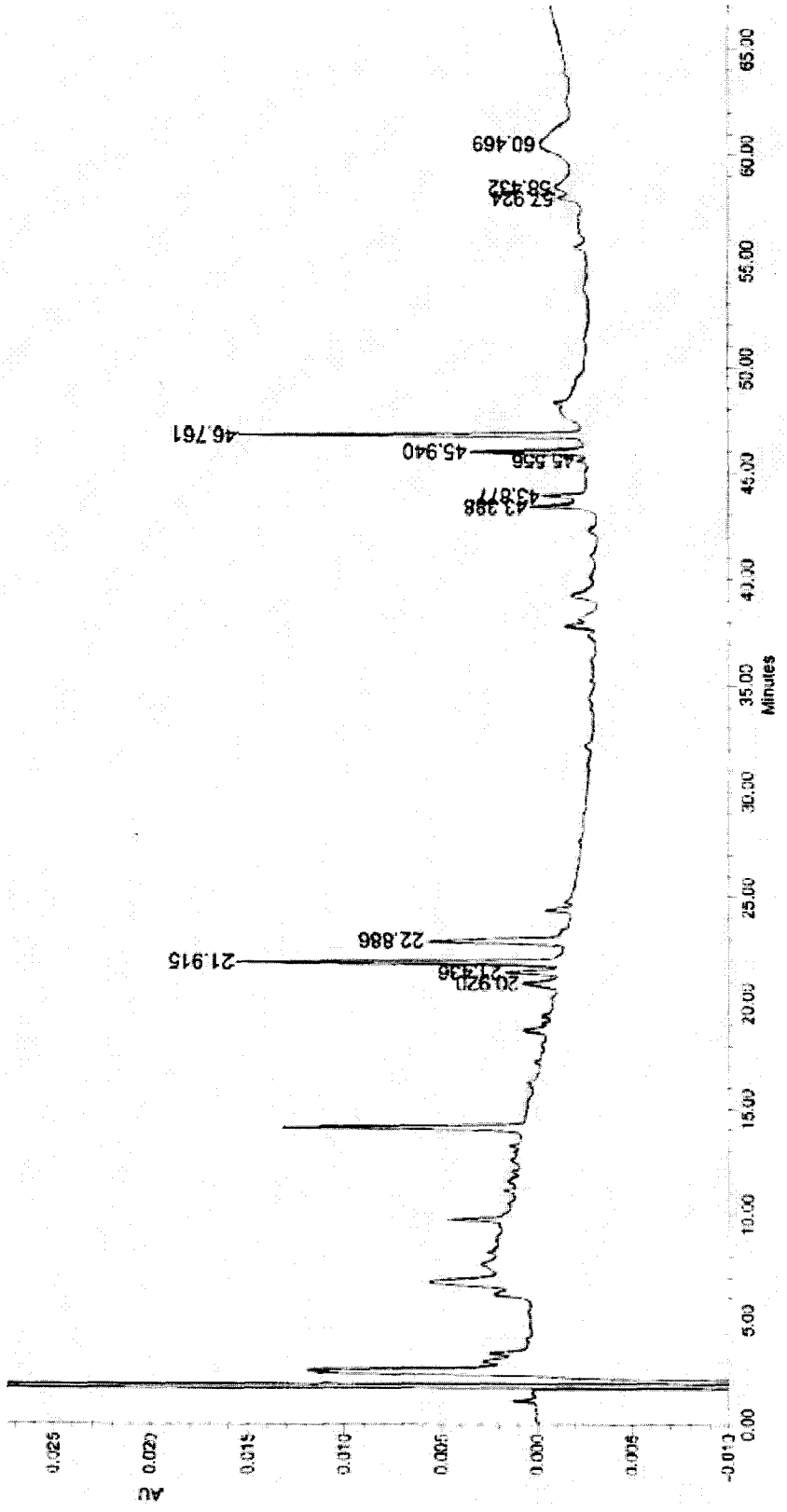
Individual MG Sample (Section II.B.1) – Male # 6



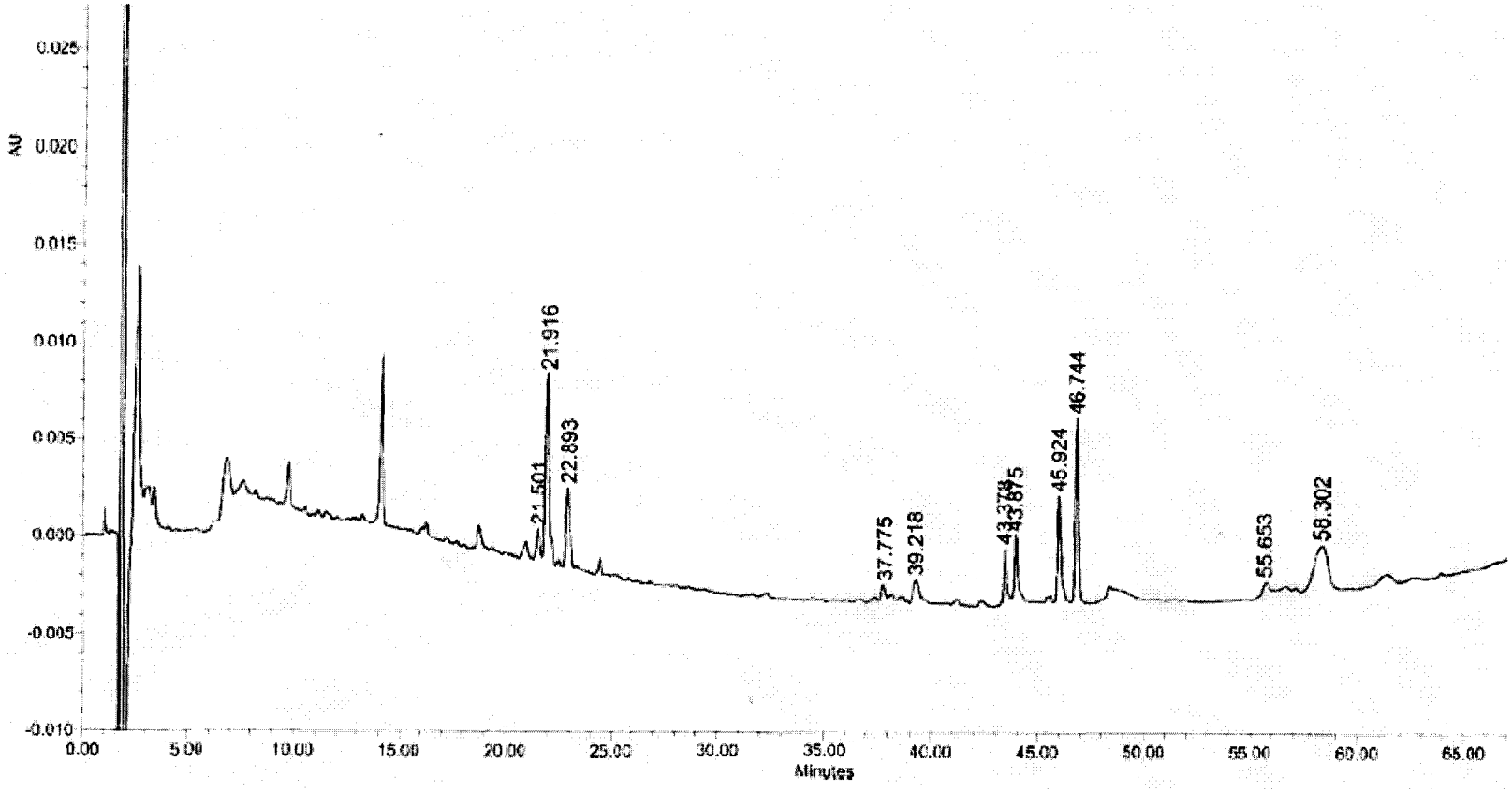
Individual MG Sample (Section II.B.1) – Male # 7



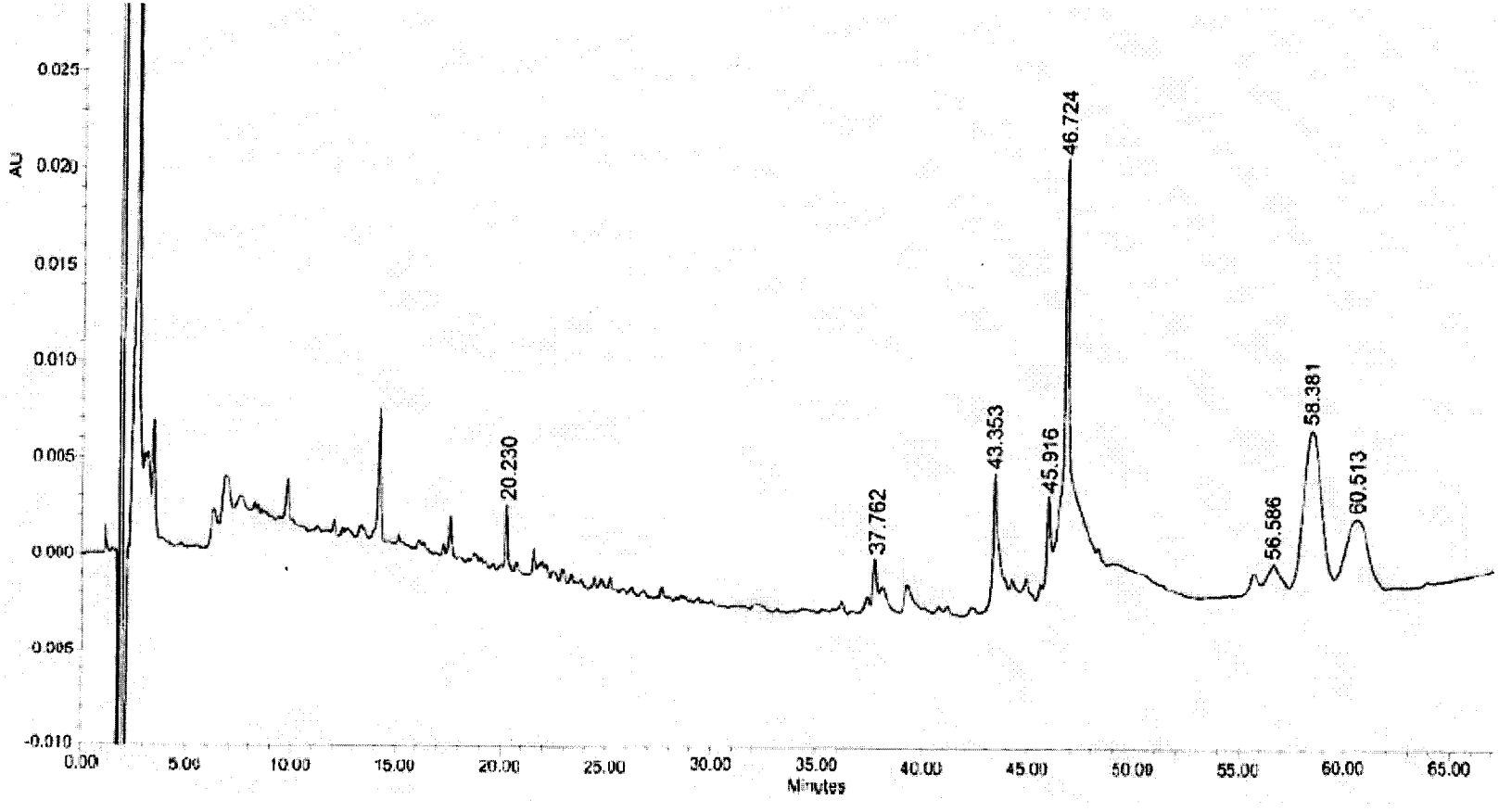
Individual MG Sample (Section II.B.1) – Male # 8



Individual MG Sample (Section II.B.1) – Male # 9

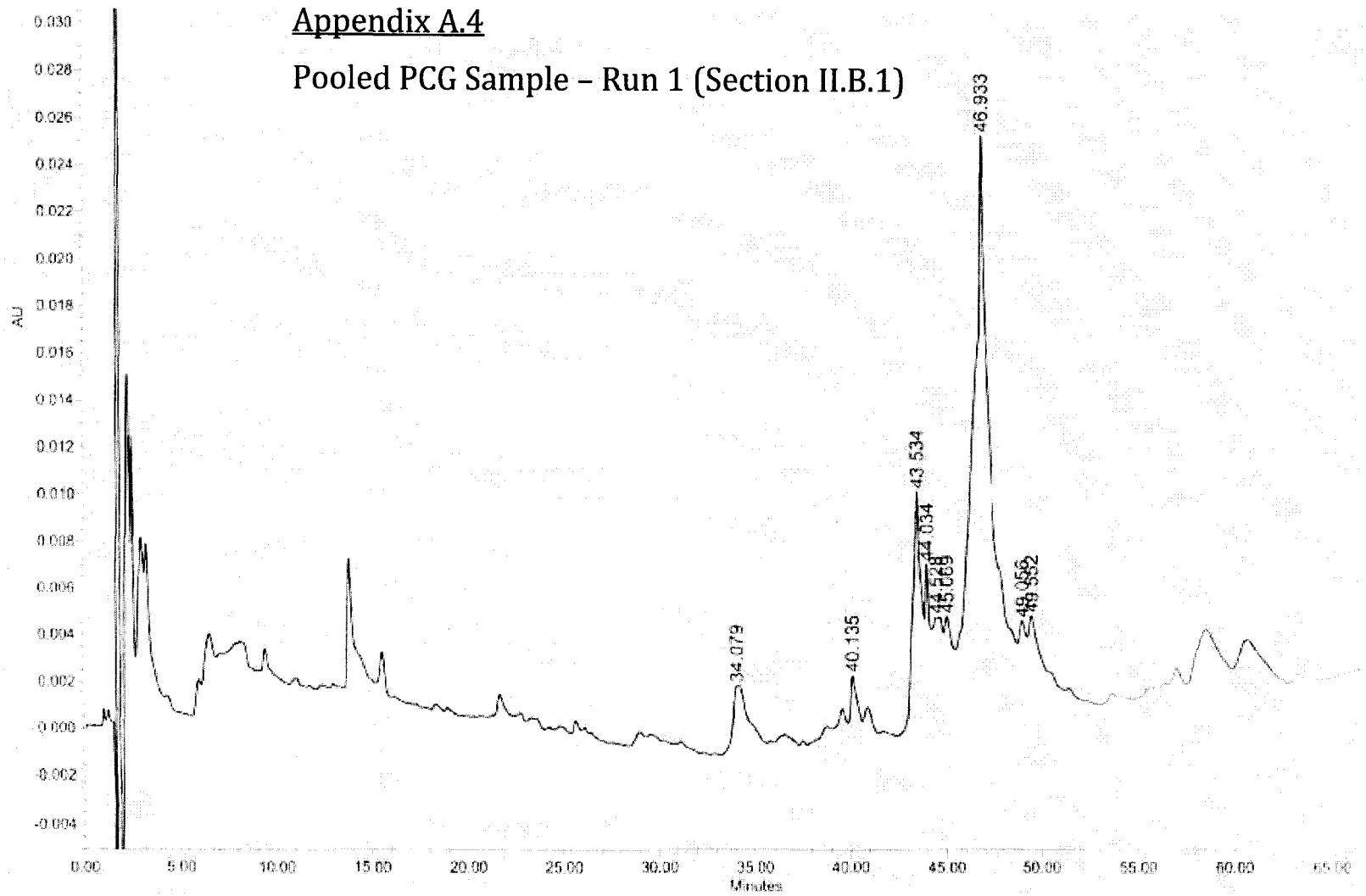


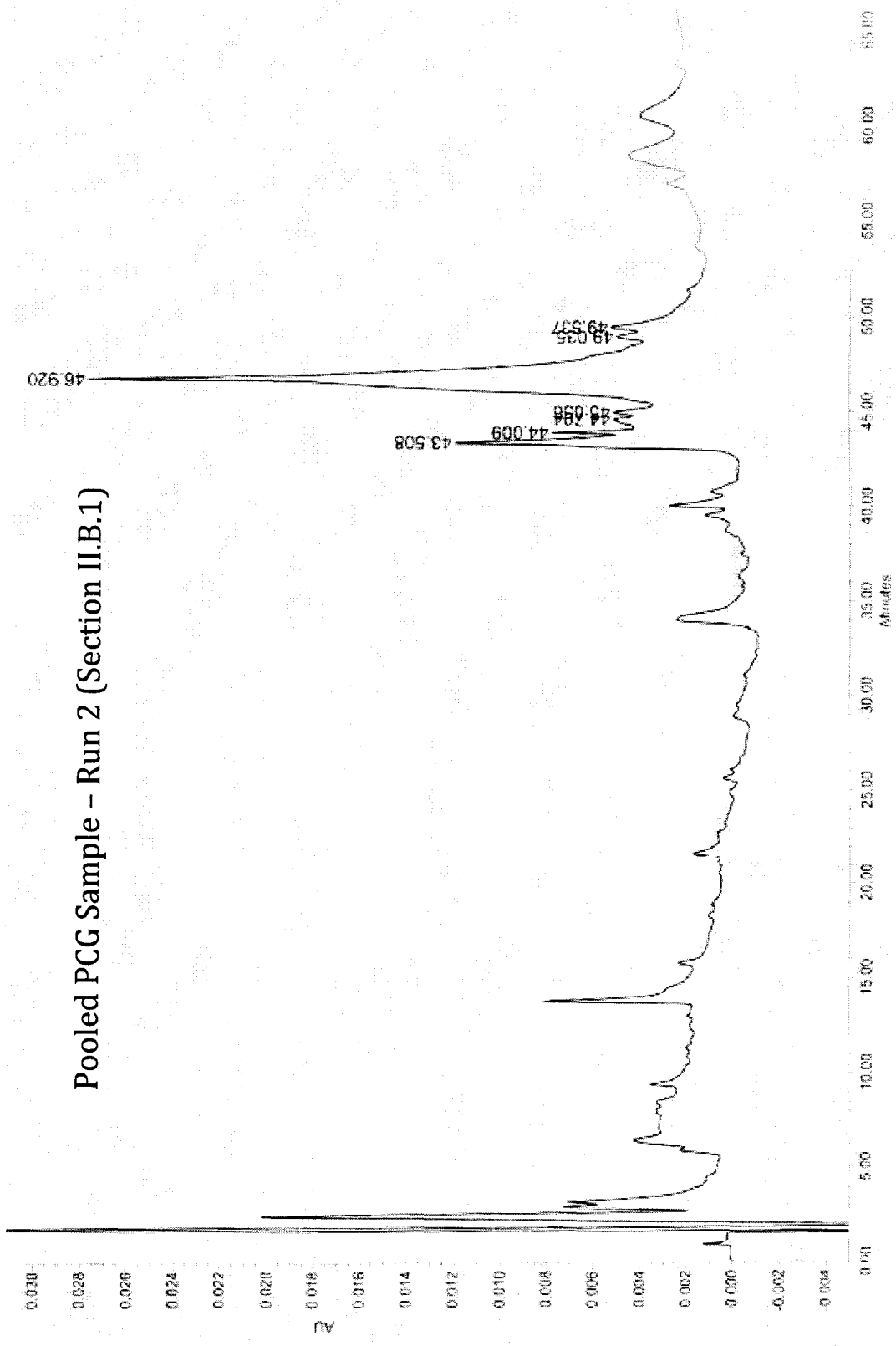
Individual MG Sample (Section II.B.1) – Male # 10



Appendix A.4

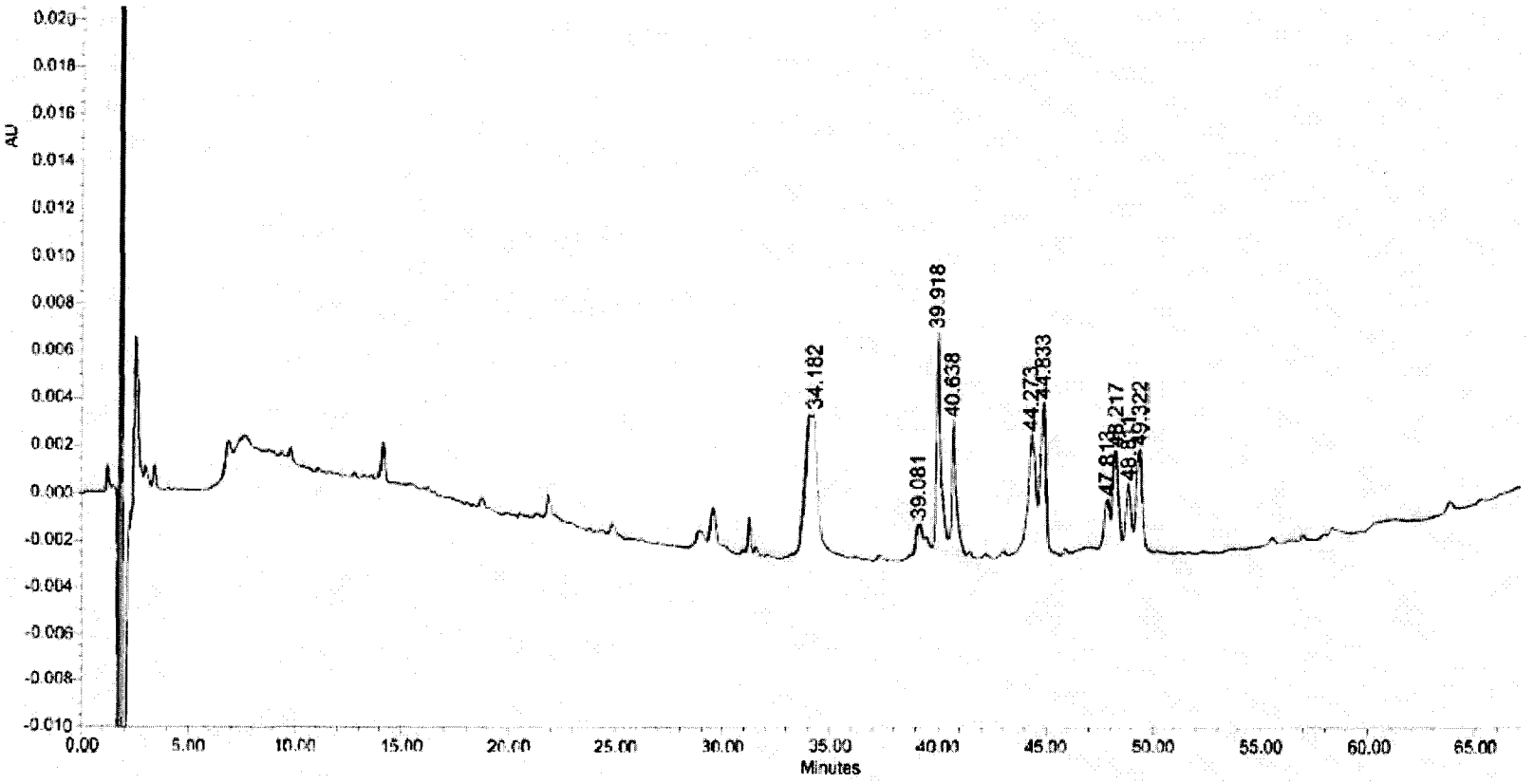
Pooled PCG Sample – Run 1 (Section II.B.1)



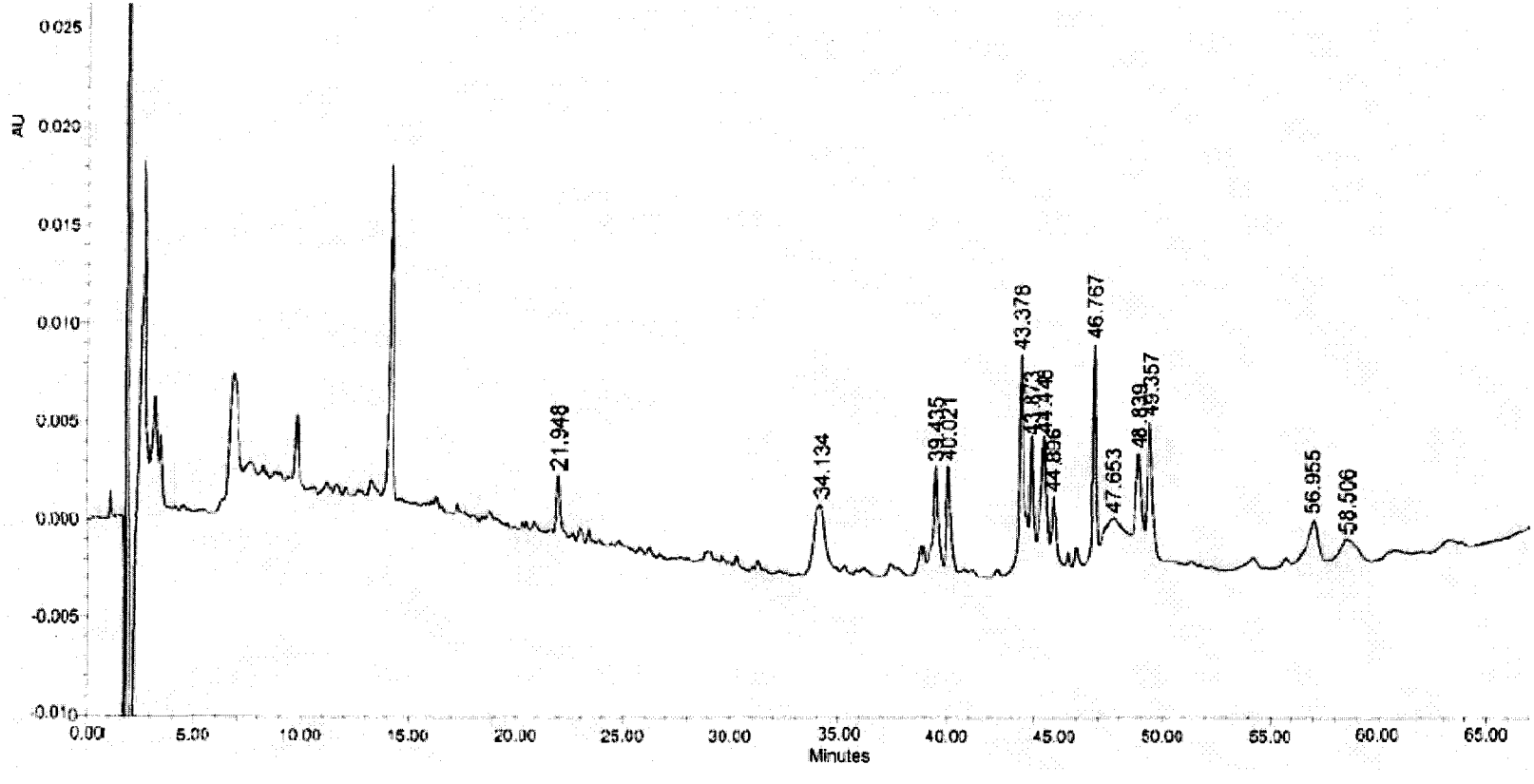

Pooled PCG Sample – Run 2 (Section II.B.1)
46.920
43.508
44.009
AU
Minutes

Appendix A.5

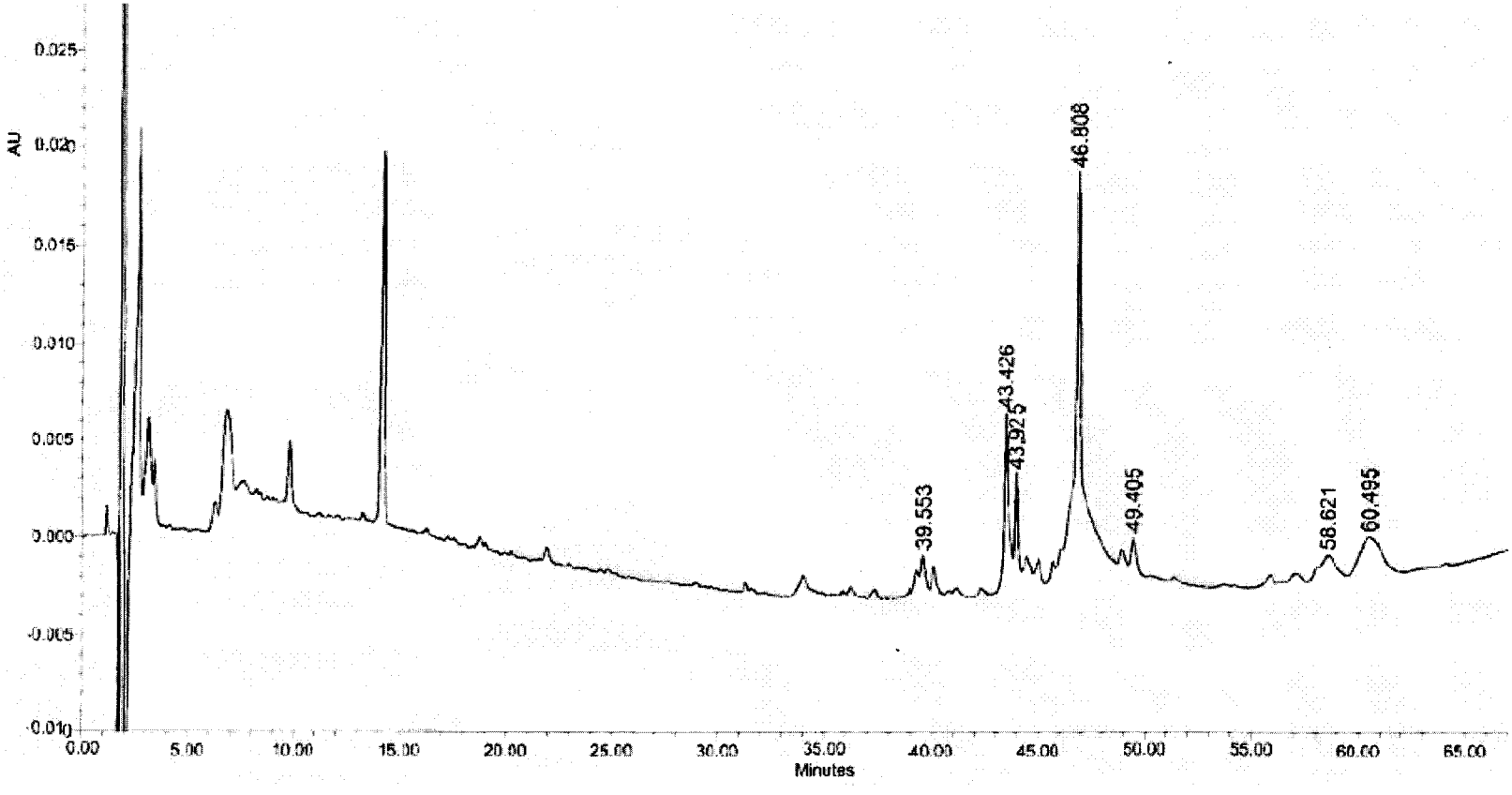
Individual PCG Sample (Section II.B.1) – Male # 1



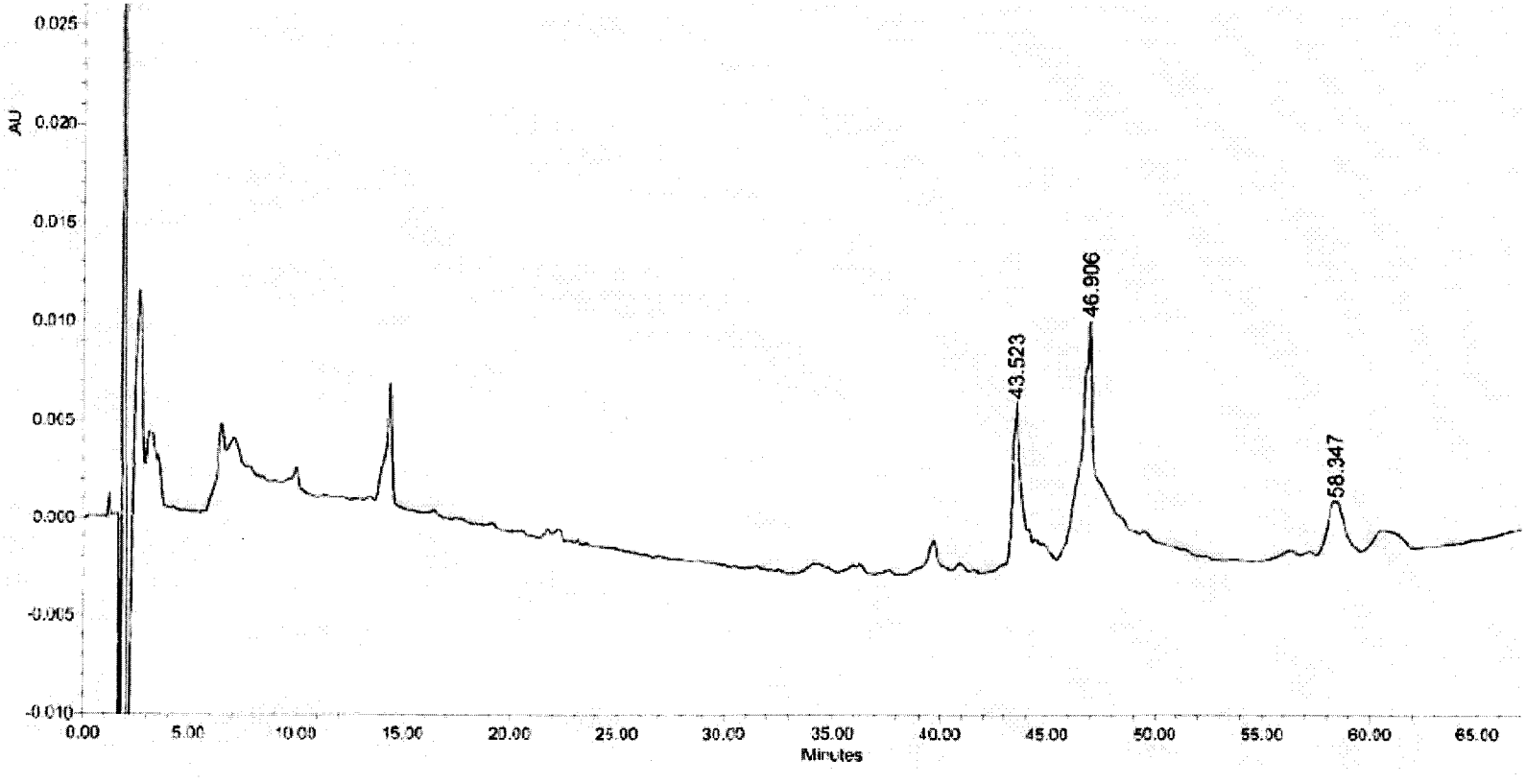
Individual PCG Sample (Section II.B.1) – Male # 2



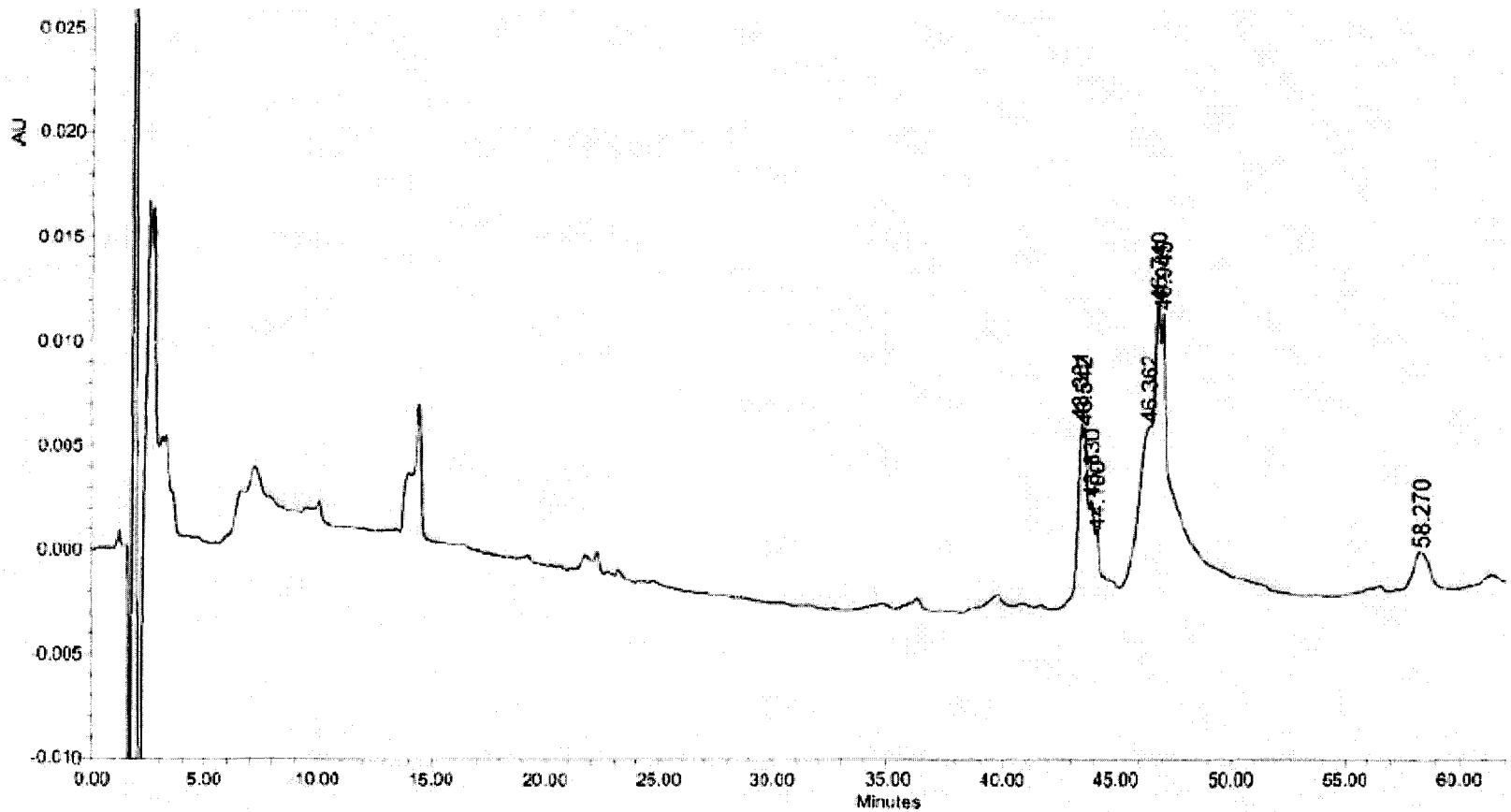
Individual PCG Sample (Section II.B.1) – Male # 3



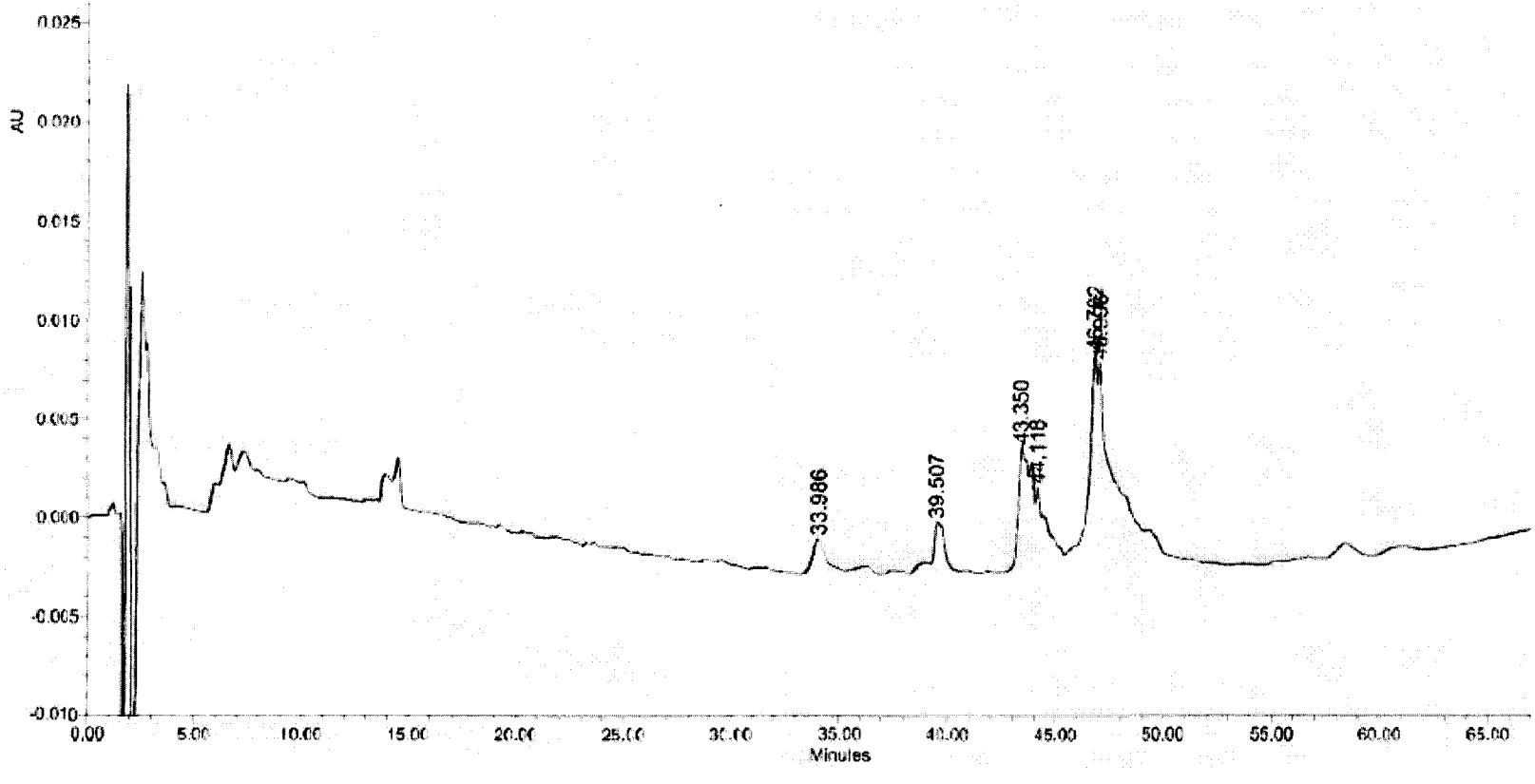
Individual PCG Sample (Section II.B.1) – Male # 4



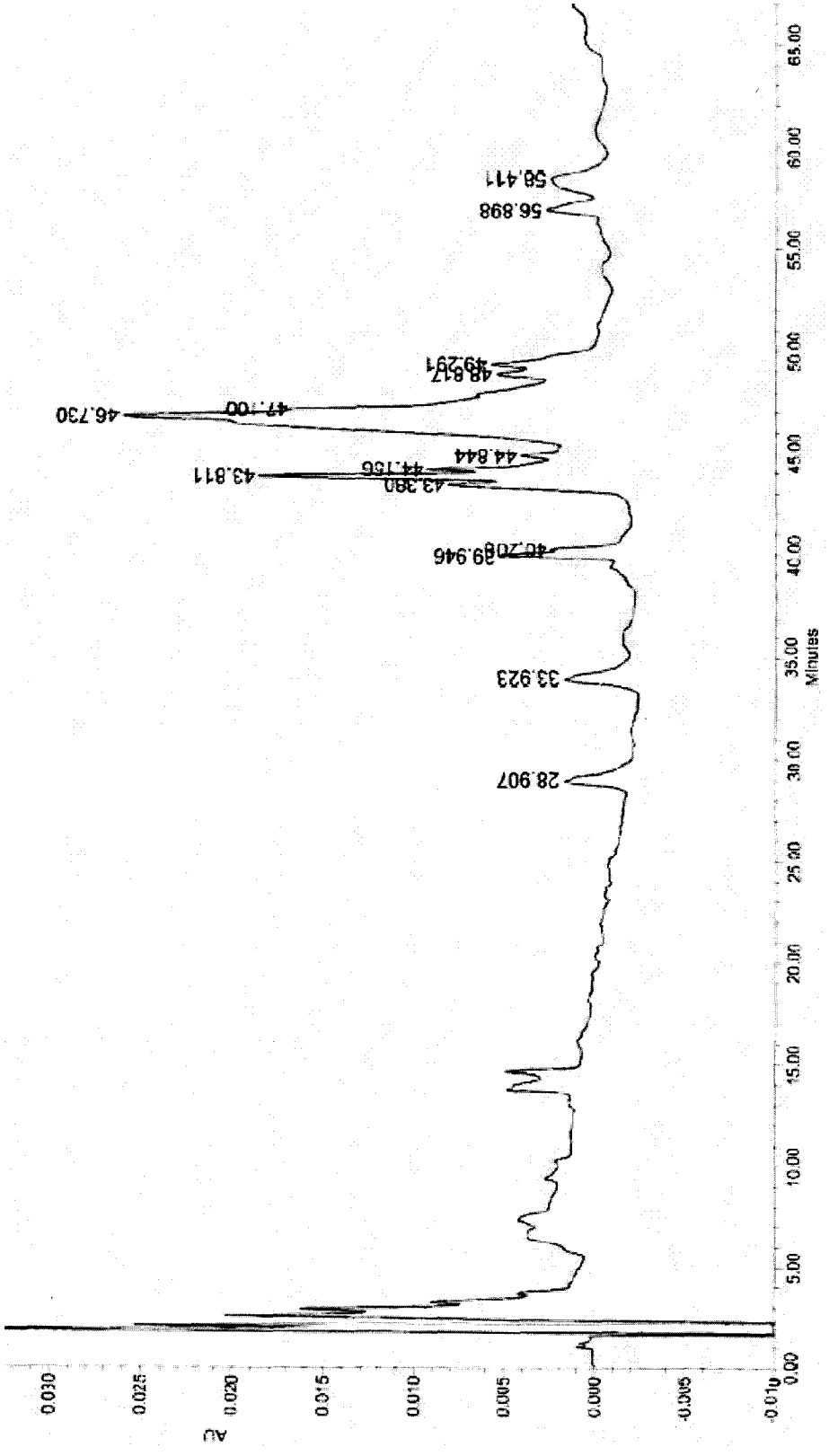
Individual PCG Sample (Section II.B.1) – Male # 5



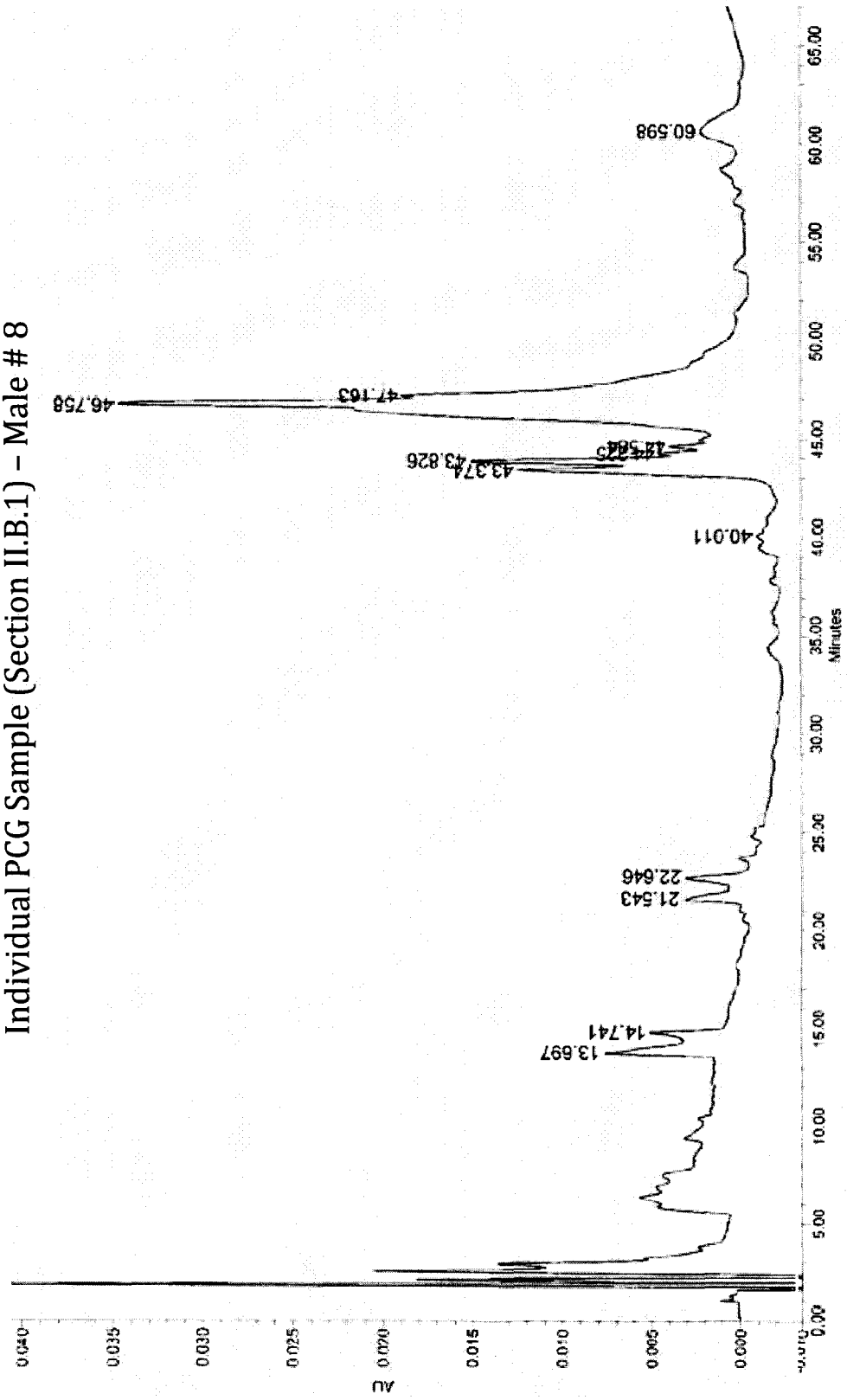
Individual PCG Sample (Section II.B.1) – Male # 6



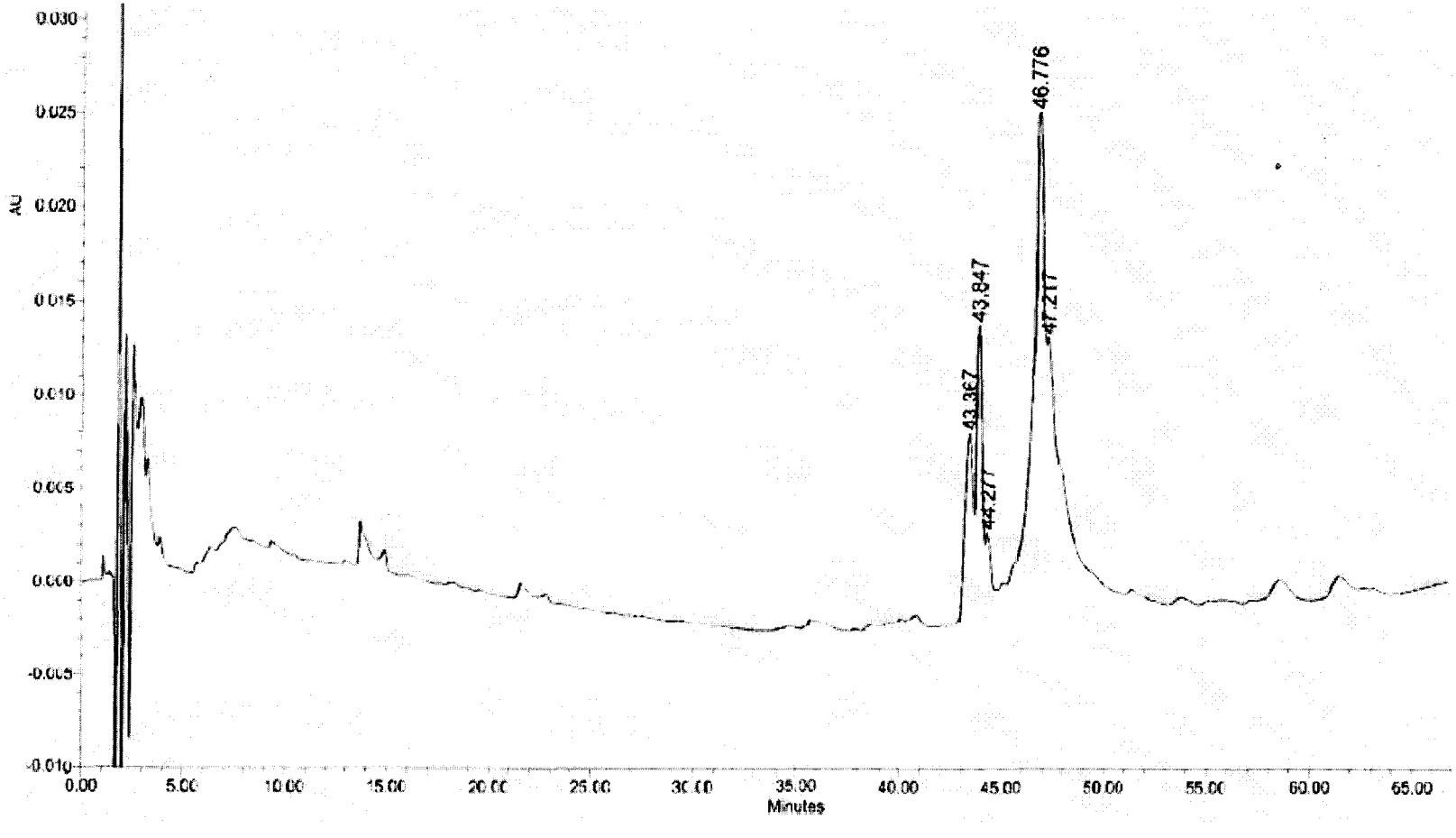
Individual PCG Sample (Section II.B.1) – Male # 7

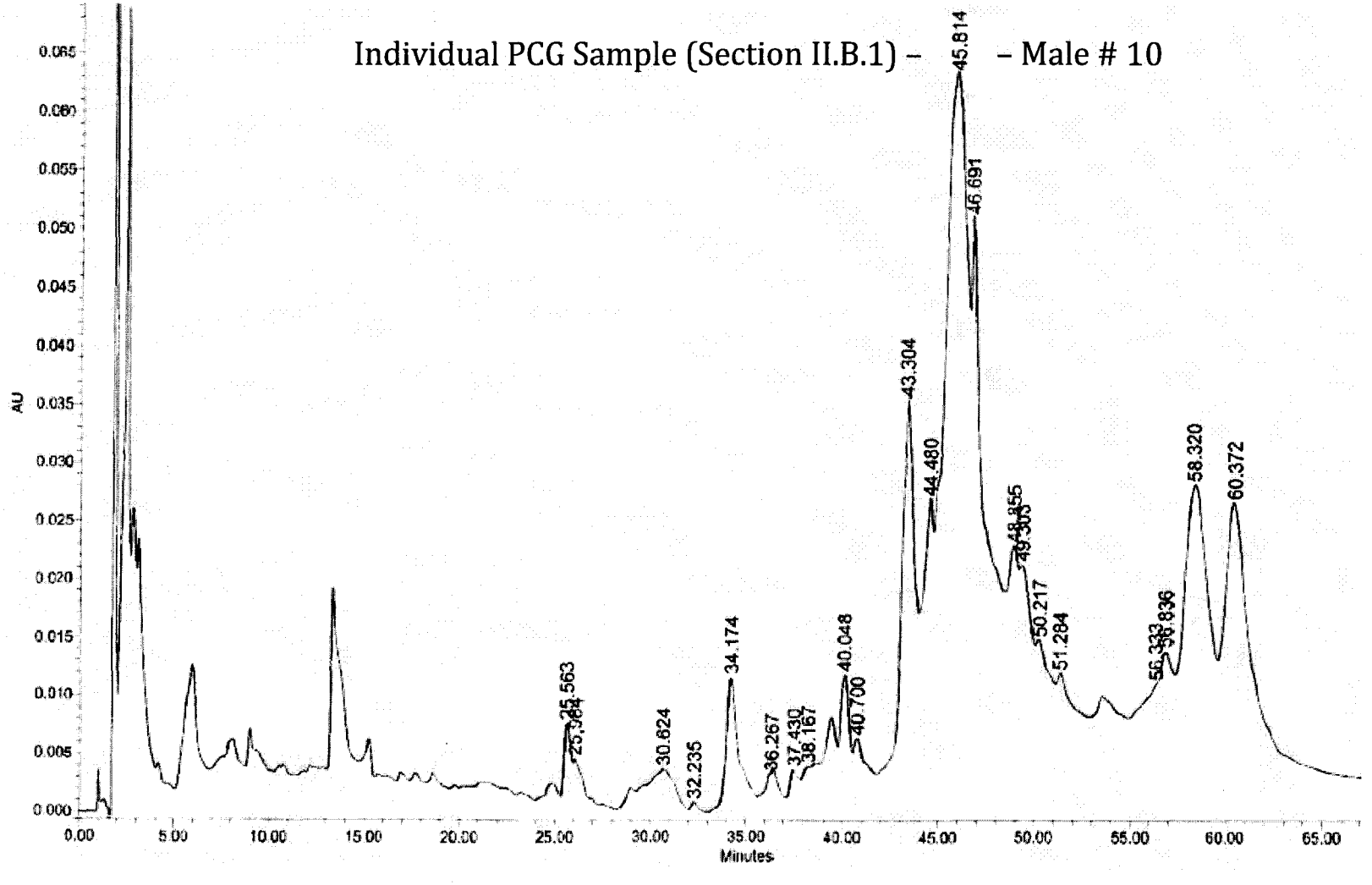


Individual PCG Sample (Section II.B.1) – Male # 8



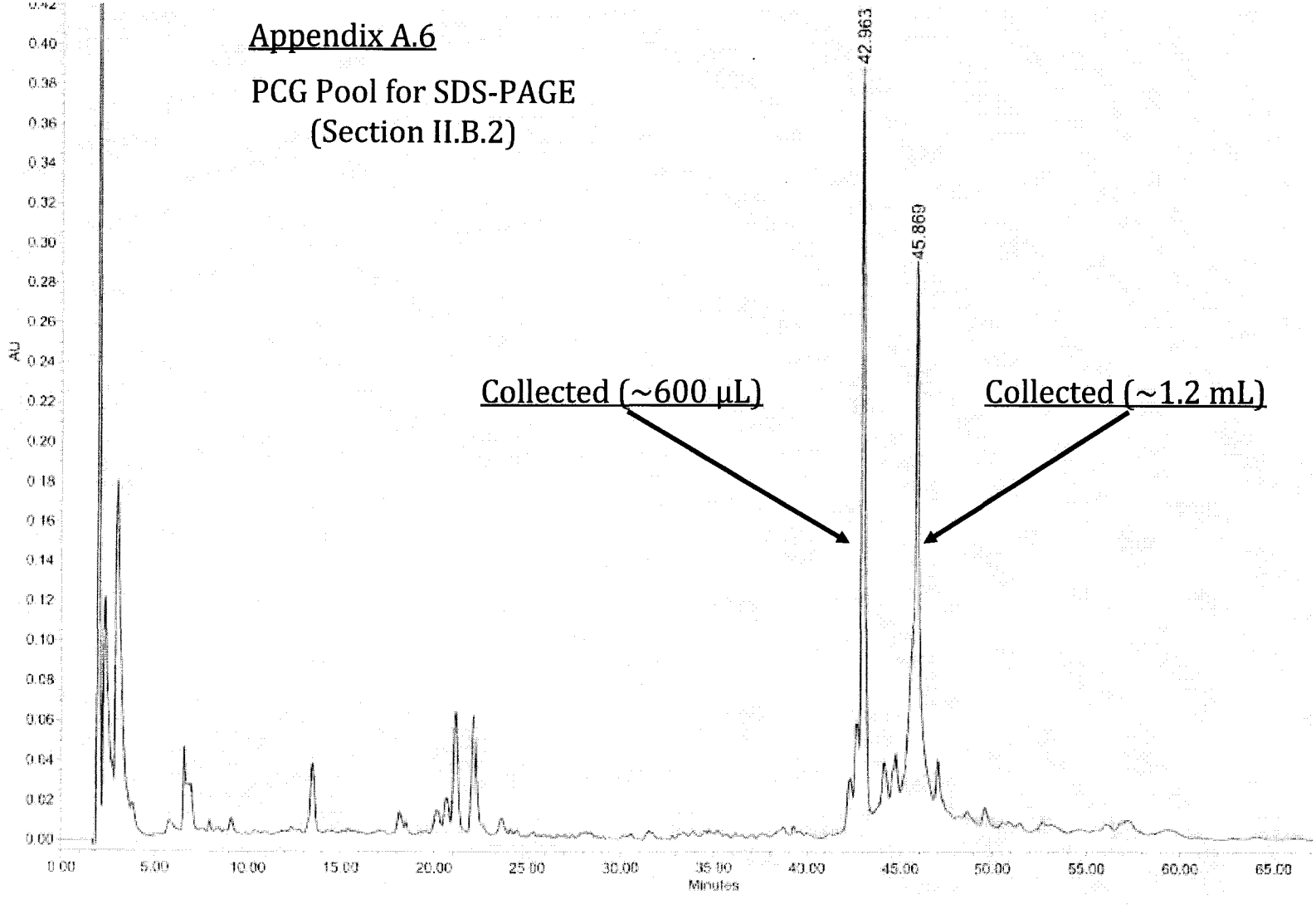
Individual PCG Sample (Section II.B.1) – Male # 9




Individual PCG Sample (Section II.B.1) – – Male # 10
AU
Minutes
0.065
0.060
0.055
0.050
0.045
0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
0.00
5.00
10.00
15.00
20.00
25.00
30.00
35.00
40.00
45.00
50.00
55.00
60.00
65.00
45.814
46.691
43.304
44.480
58.320
60.372
34.174
40.048
40.700
30.624
32.235
36.267
37.430
38.167
50.217
51.284

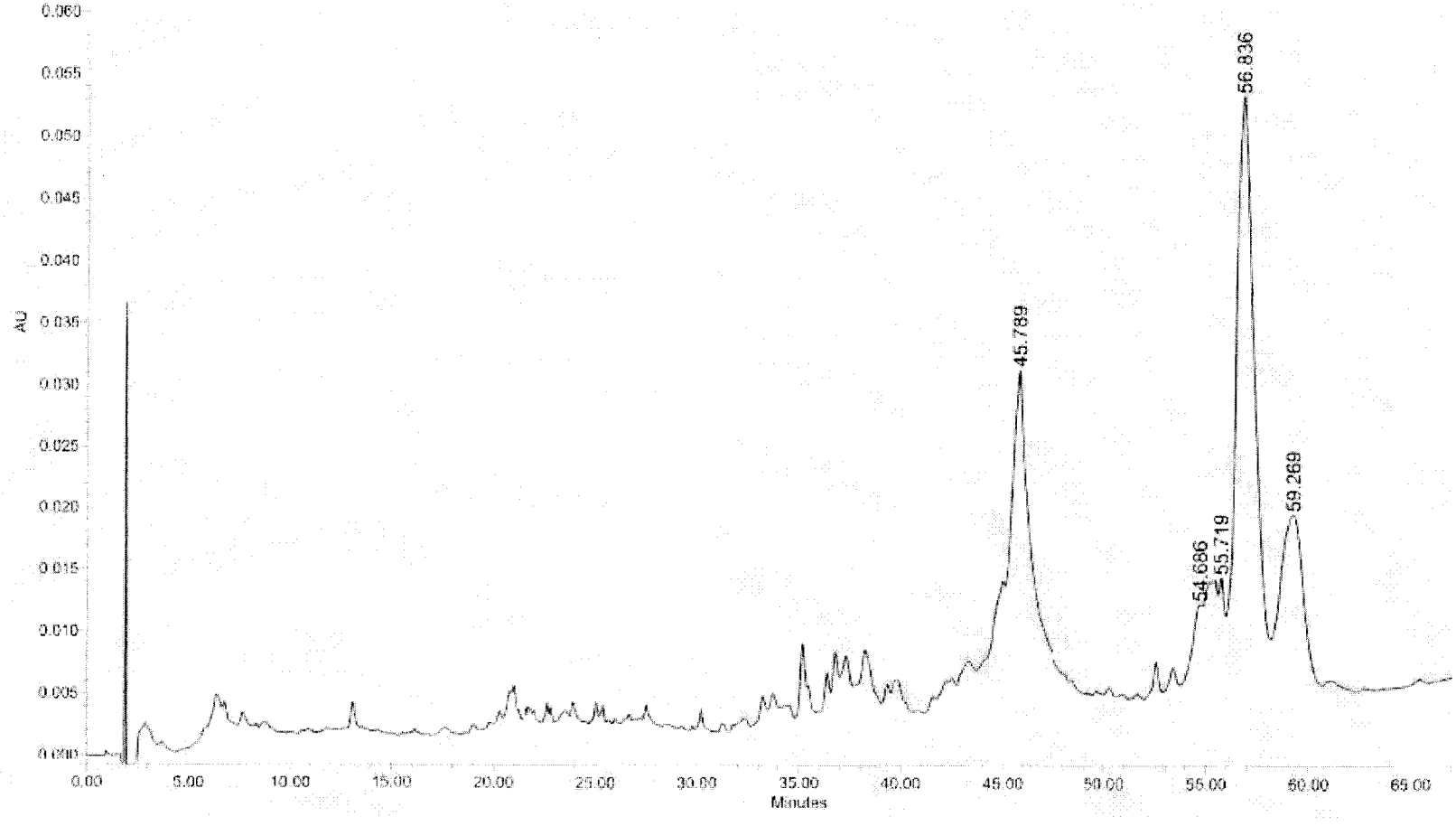
Appendix A.6

PCG Pool for SDS-PAGE
(Section II.B.2)



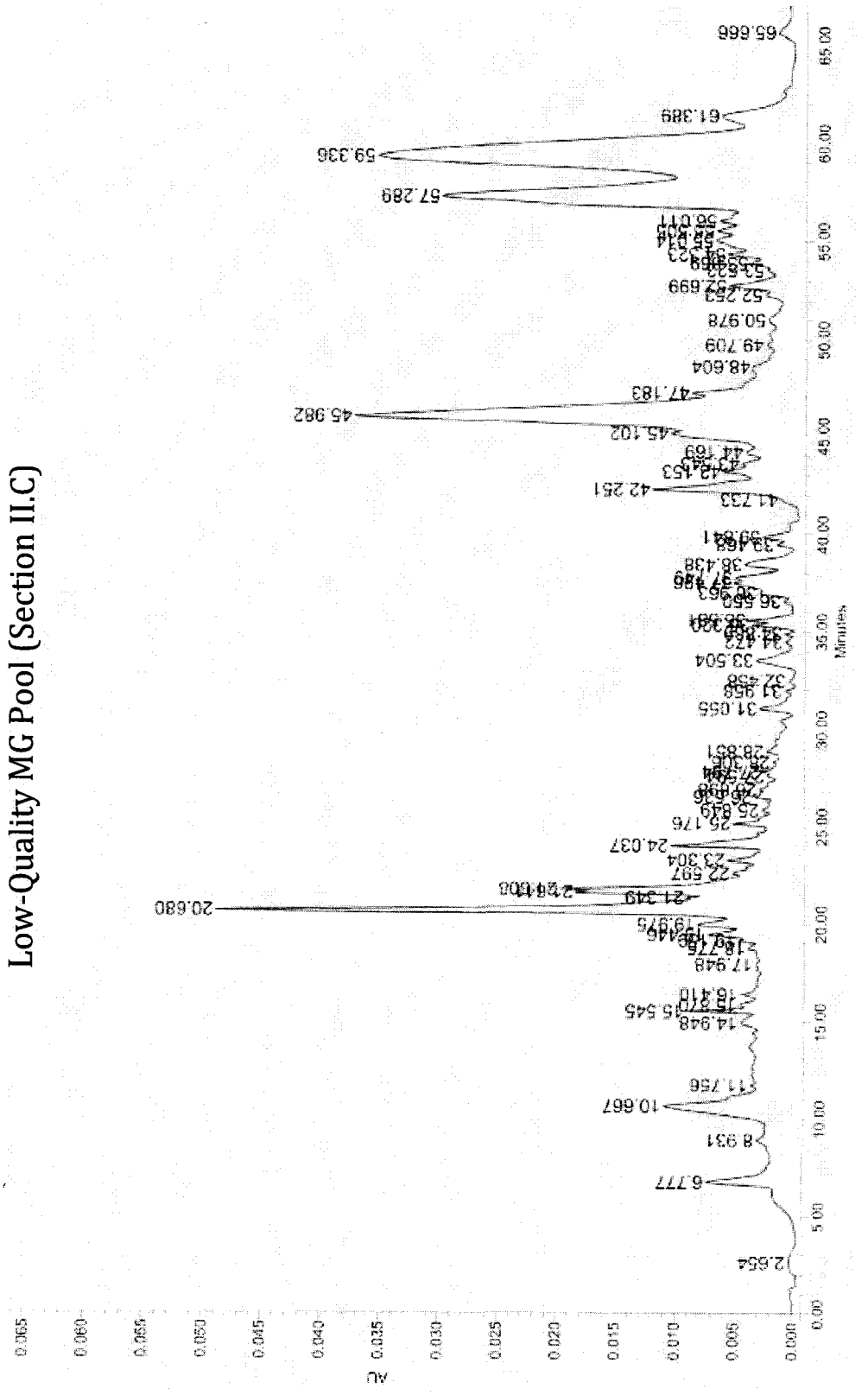
Appendix A.7

Field-Quality MG Pool (Section II.C)



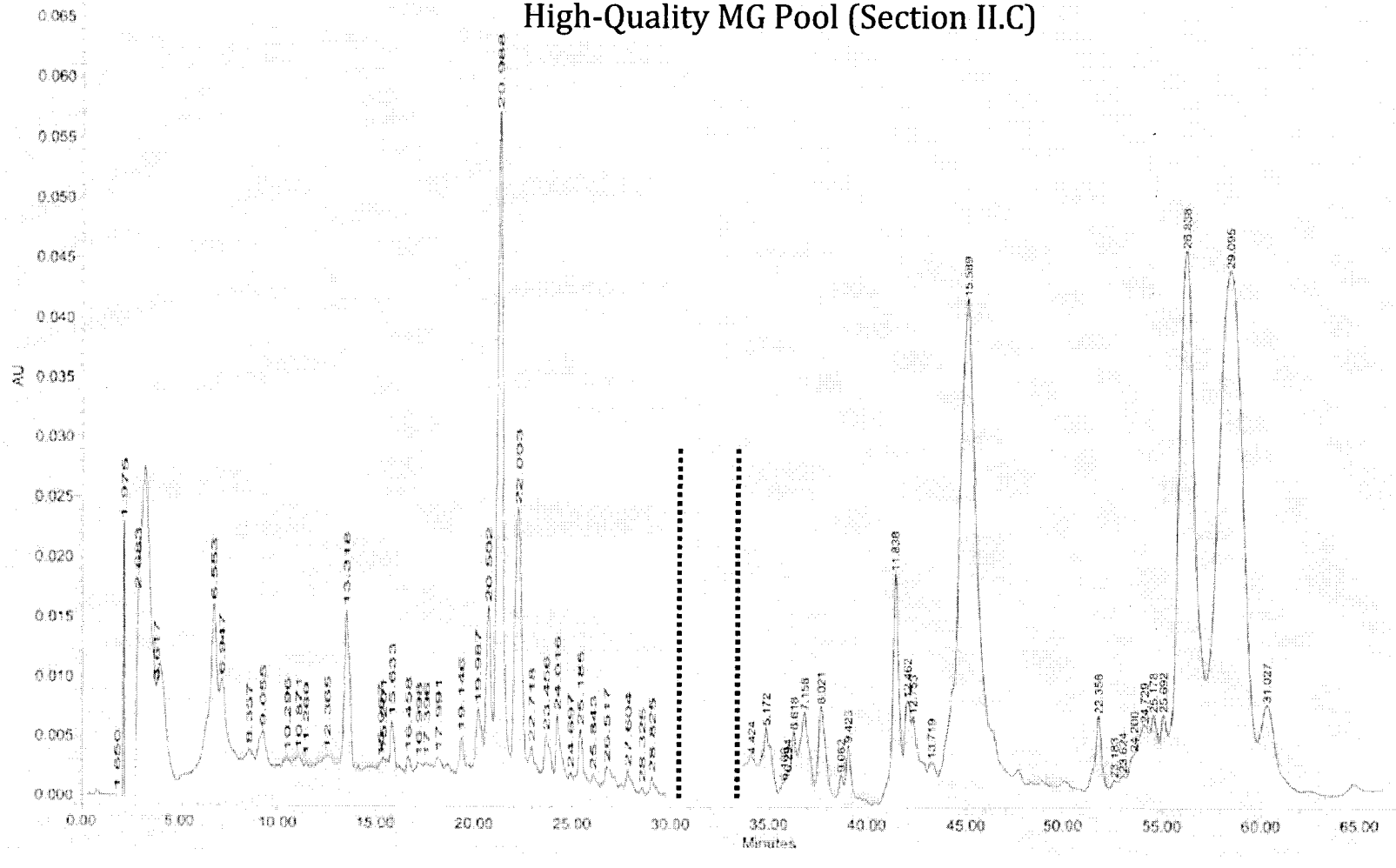
Appendix A.8

Low-Quality MG Pool (Section II.C)



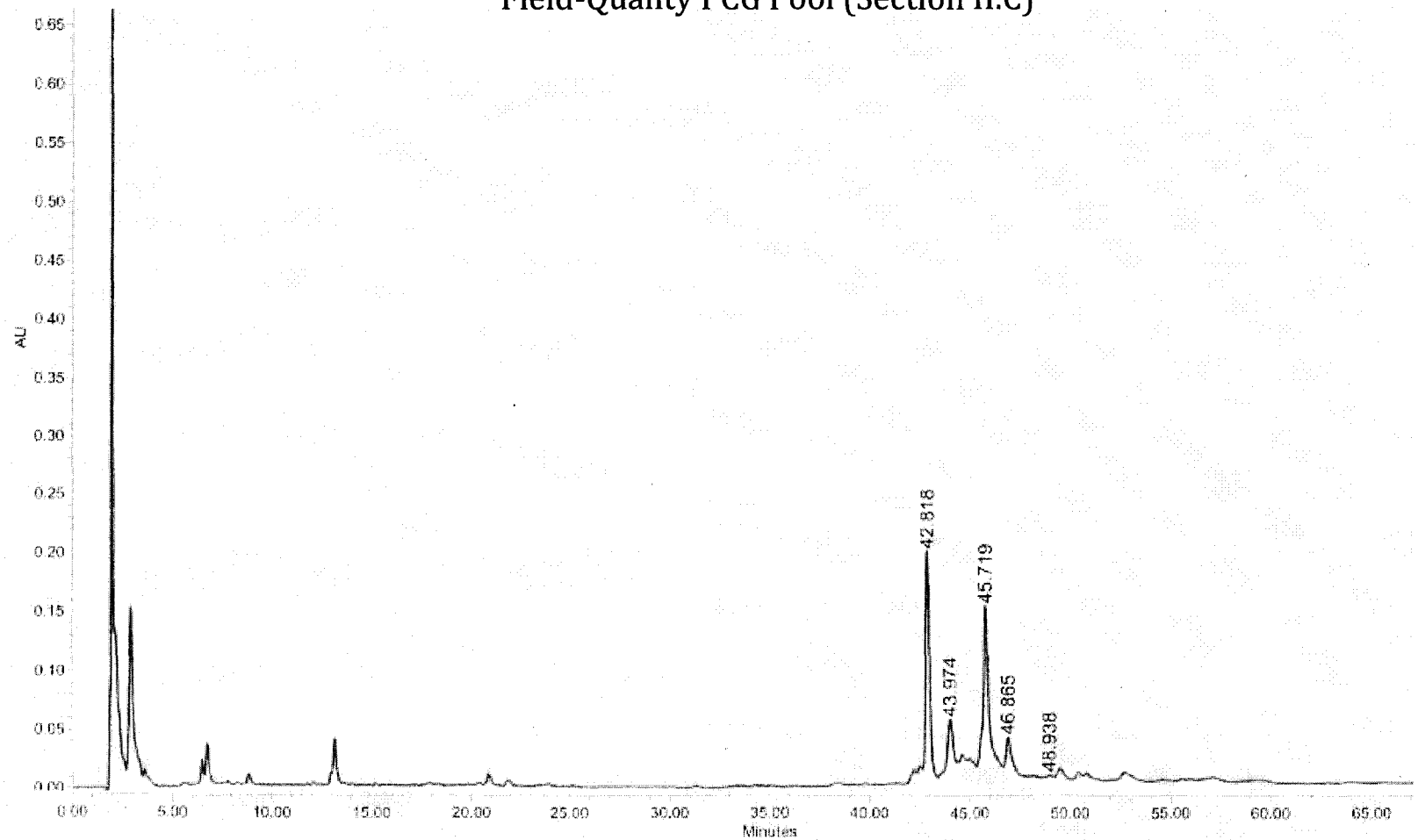
Appendix A.9

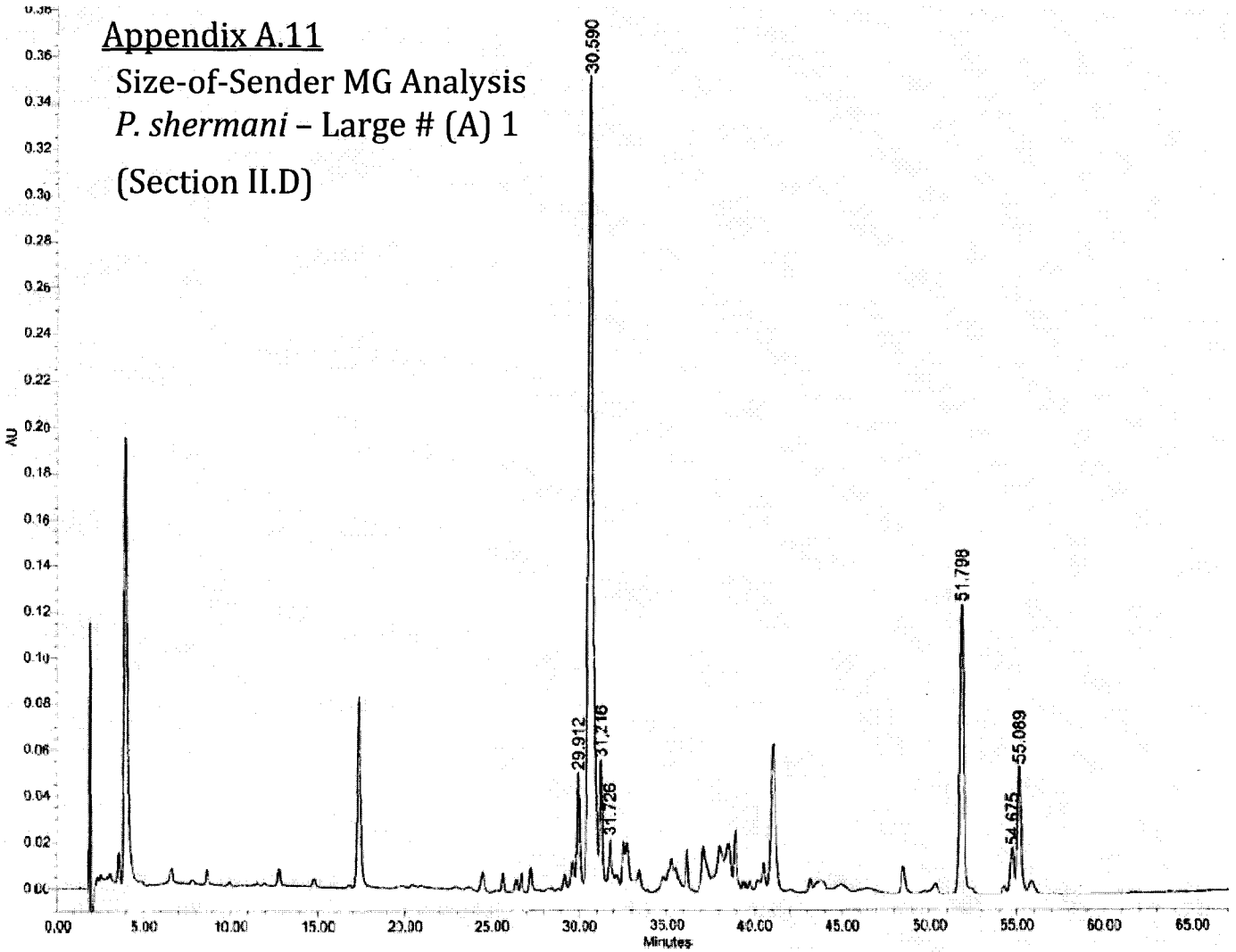
High-Quality MG Pool (Section II.C)

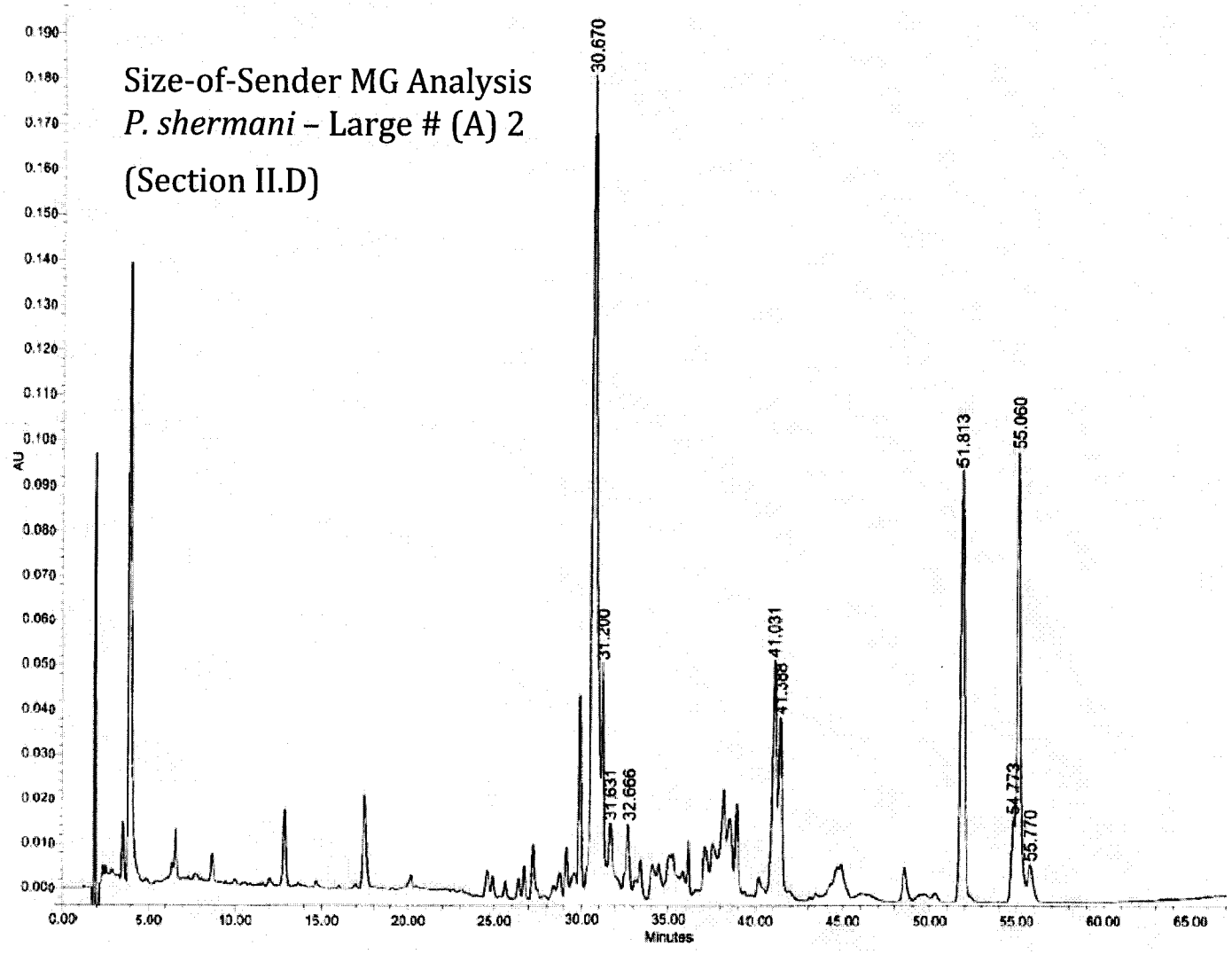


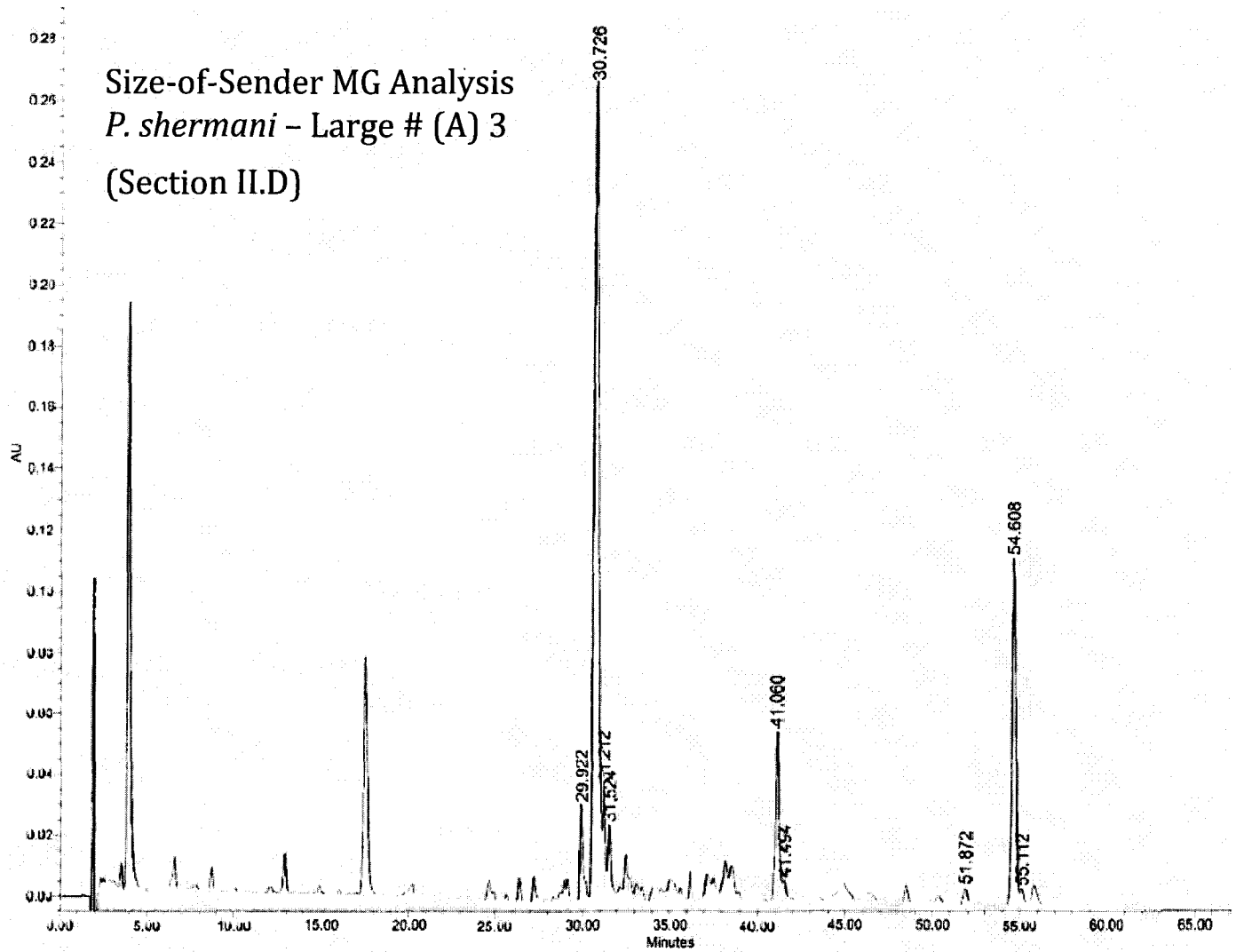
Appendix A.10

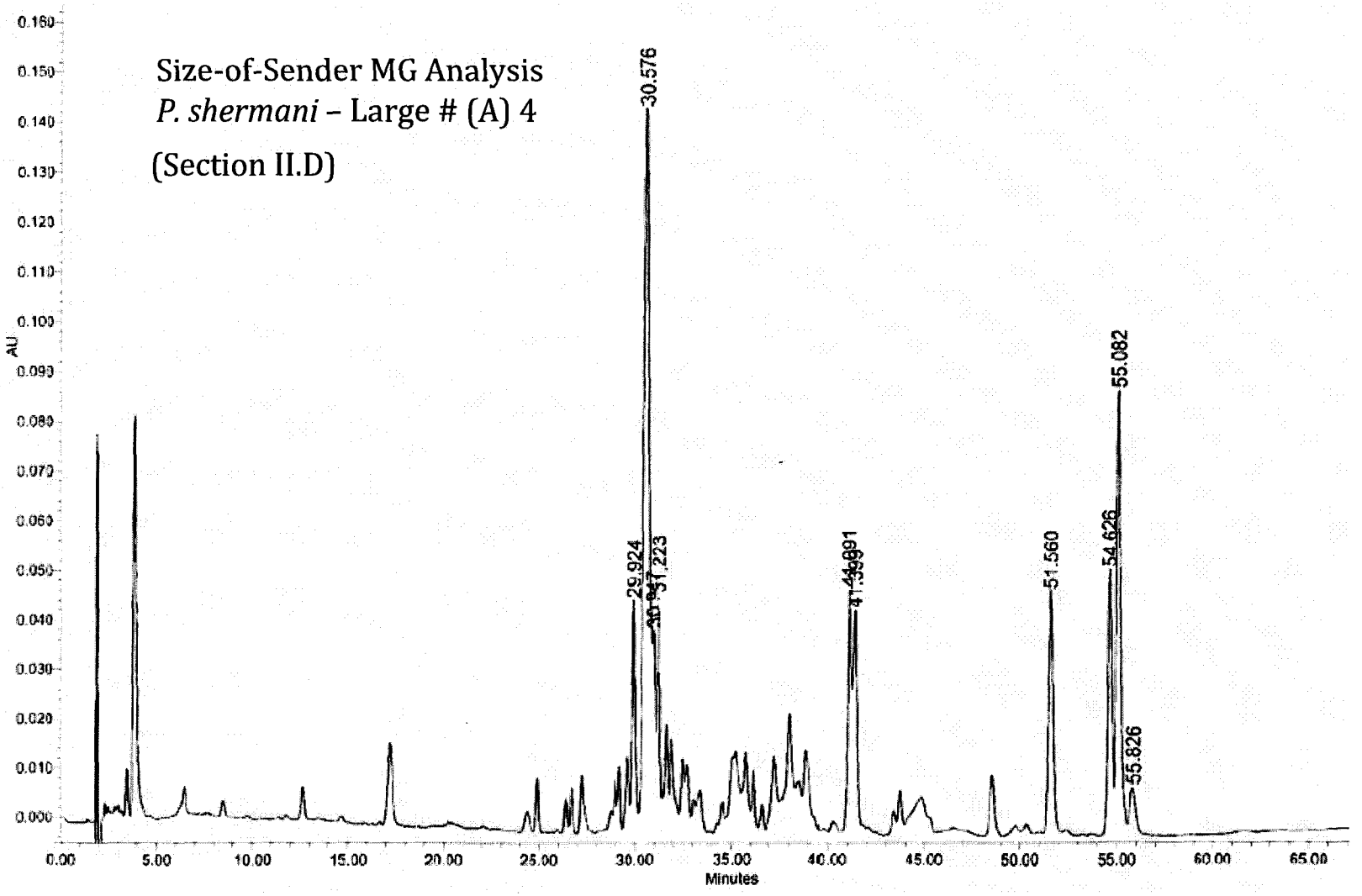
Field-Quality PCG Pool (Section II.C)



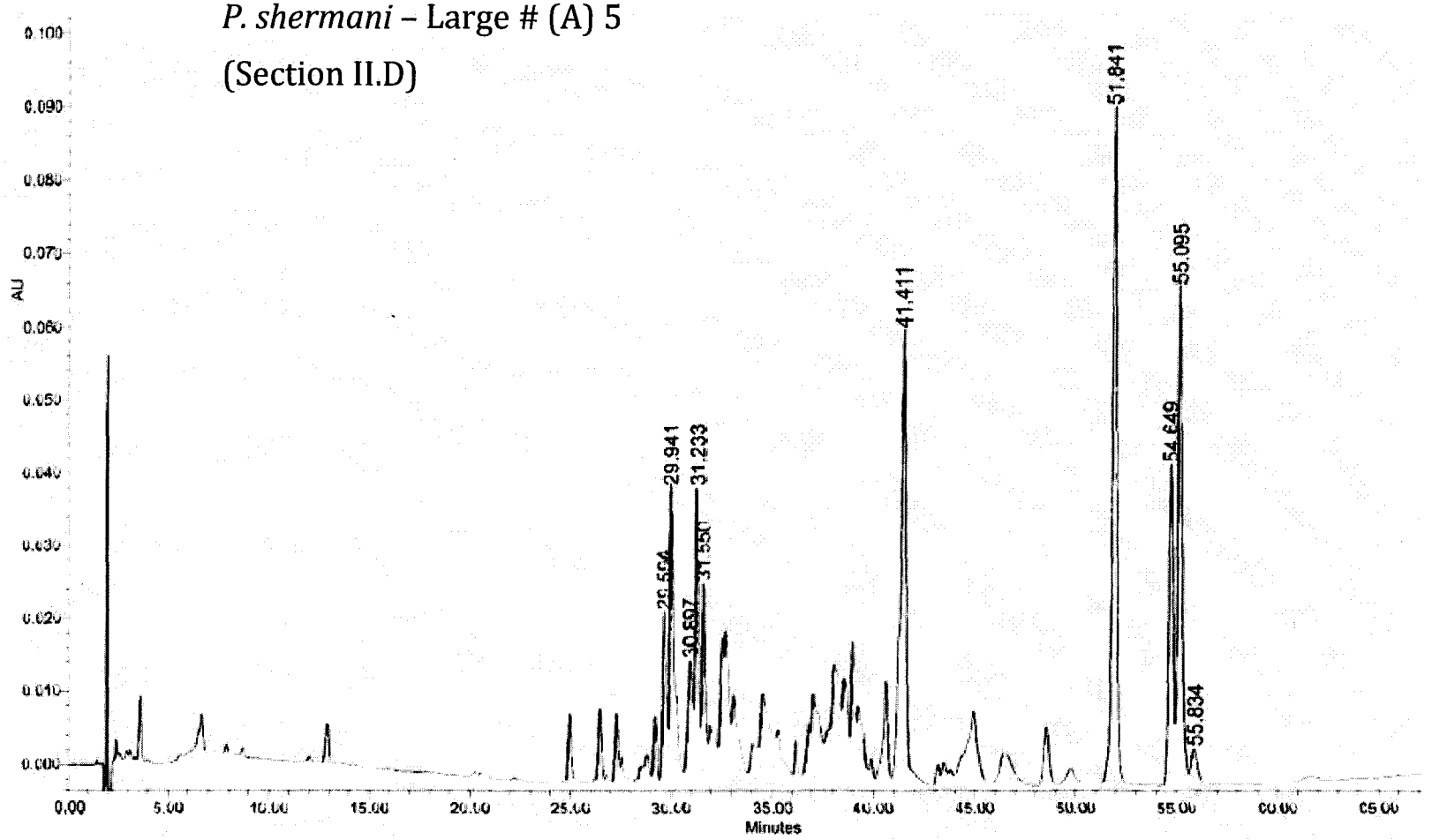

Appendix A.11
Size-of-Sender MG Analysis
P. shermani – Large # (A) 1
(Section II.D)
AU
Minutes
30.590
29.912
31.216
31.726
51.798
54.675
55.089
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
0.18
0.20
0.22
0.24
0.26
0.28
0.30
0.32
0.34
0.36
0.00
5.00
10.00
15.00
20.00
25.00
30.00
35.00
40.00
45.00
50.00
55.00
60.00
65.00


Size-of-Sender MG Analysis
P. shermani – Large # (A) 2
(Section II.D)
AU
Minutes
30.670
31.200
31.631
32.666
41.031
41.388
51.813
54.723
55.060
55.770

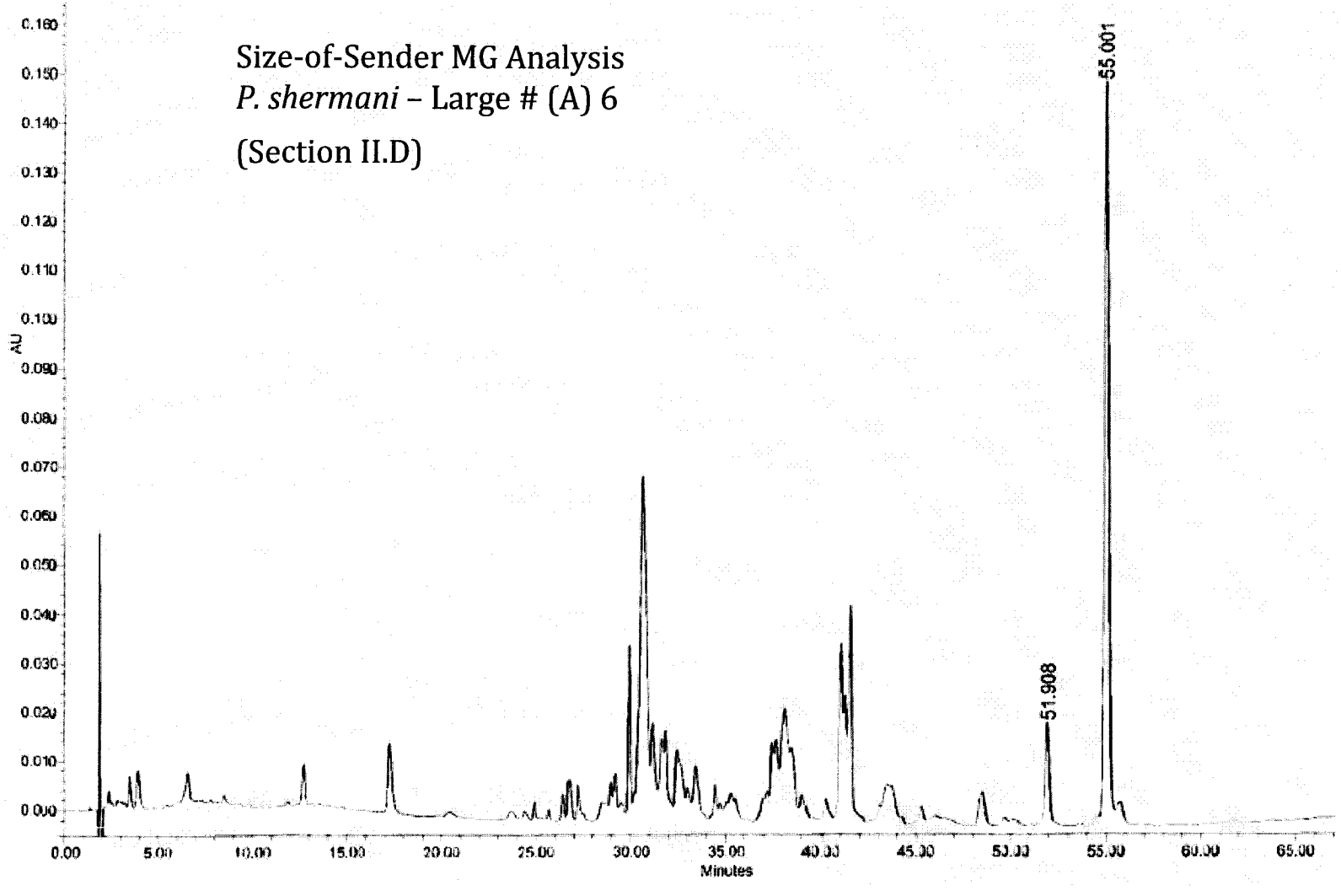

Size-of-Sender MG Analysis
P. shermani – Large # (A) 3
(Section II.D)
AU
Minutes
30.726
29.922
41.060
54.608
51.872
0.00
5.00
10.00
15.00
20.00
25.00
30.00
35.00
40.00
45.00
50.00
55.00
60.00
65.00

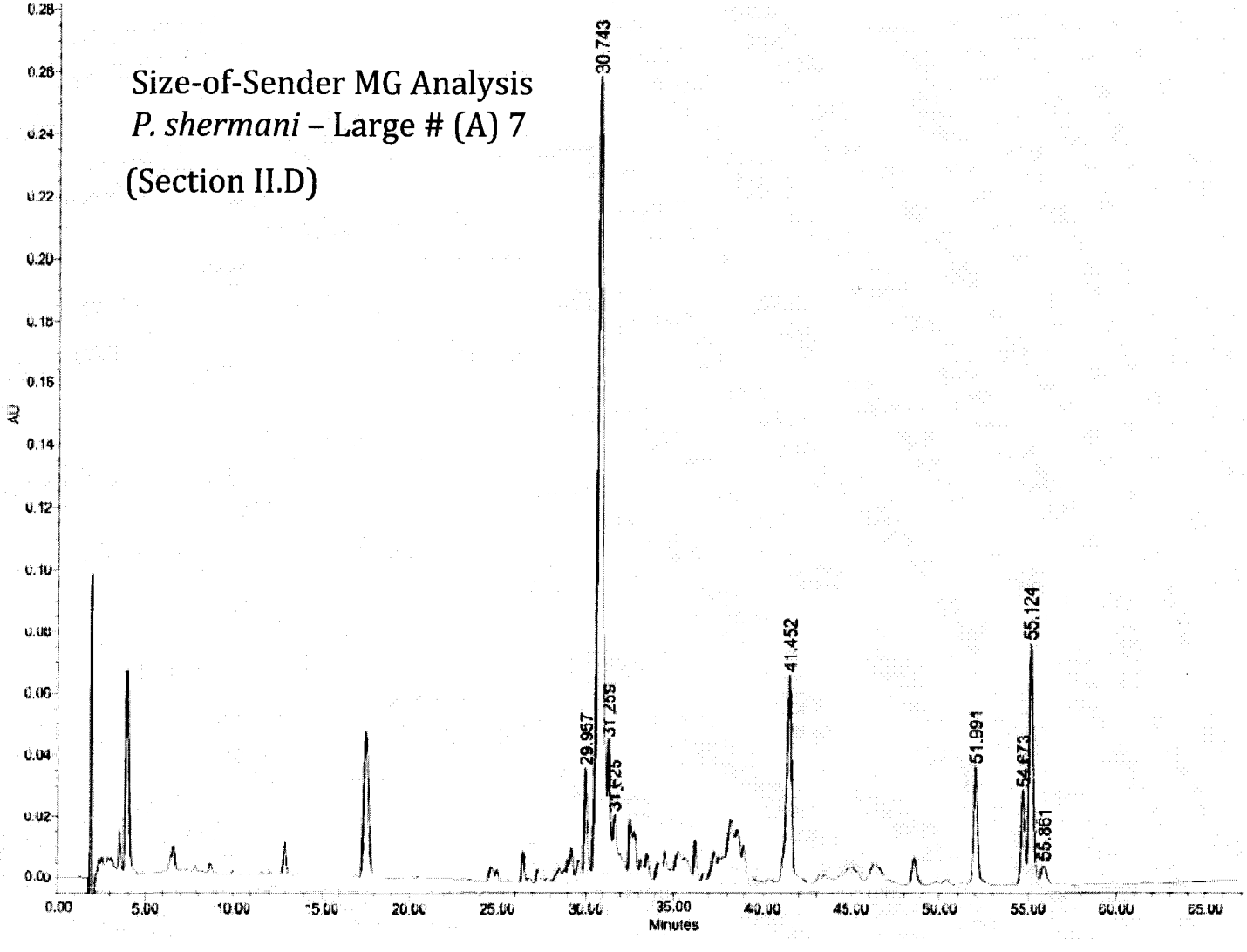

Size-of-Sender MG Analysis
P. shermani – Large # (A) 4
(Section II.D)
AU
Minutes
30.576
29.924
55.082
54.626
51.560
55.826

Size-of-Sender MG Analysis
P. shermani – Large # (A) 5
(Section II.D)

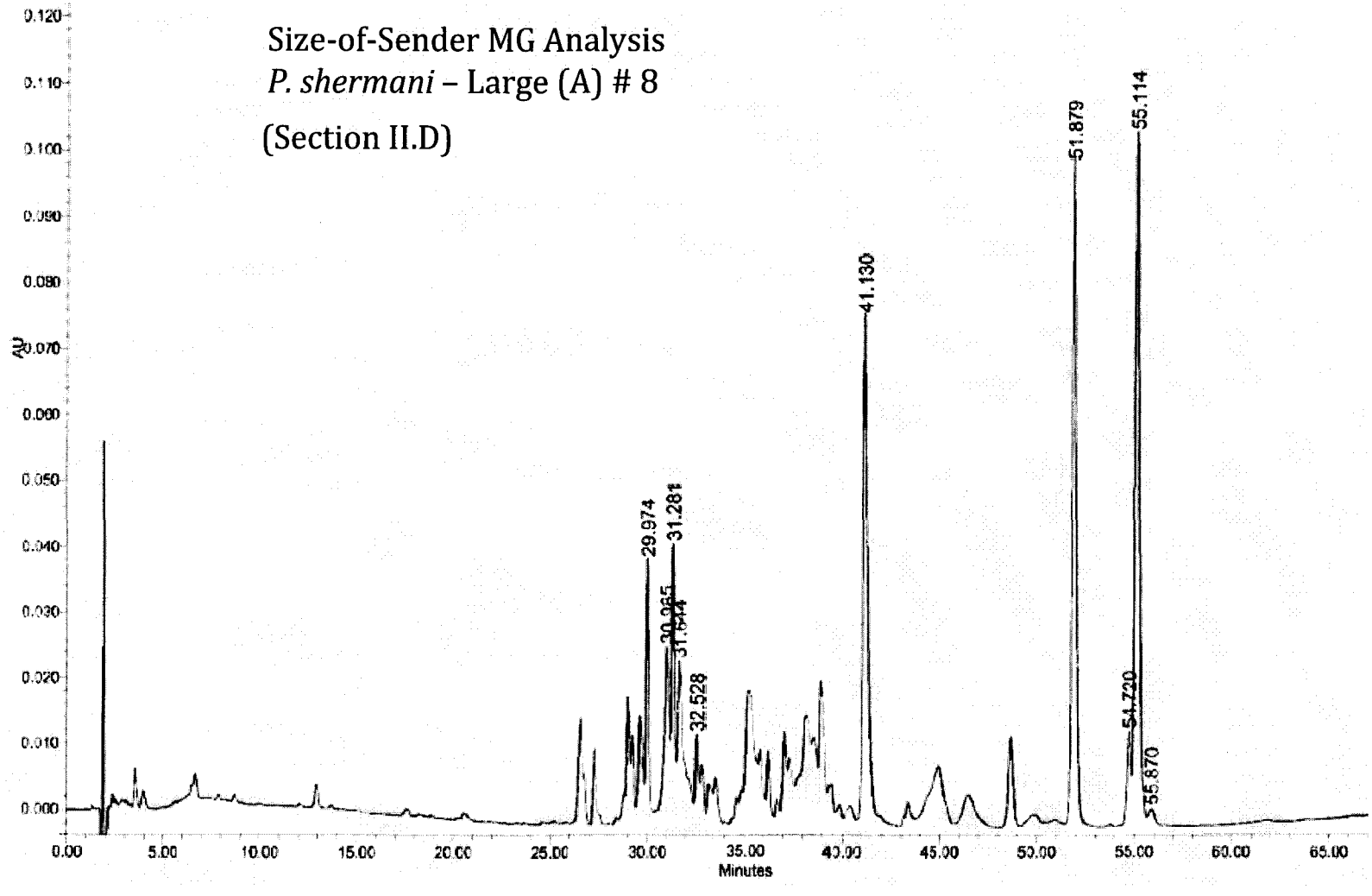


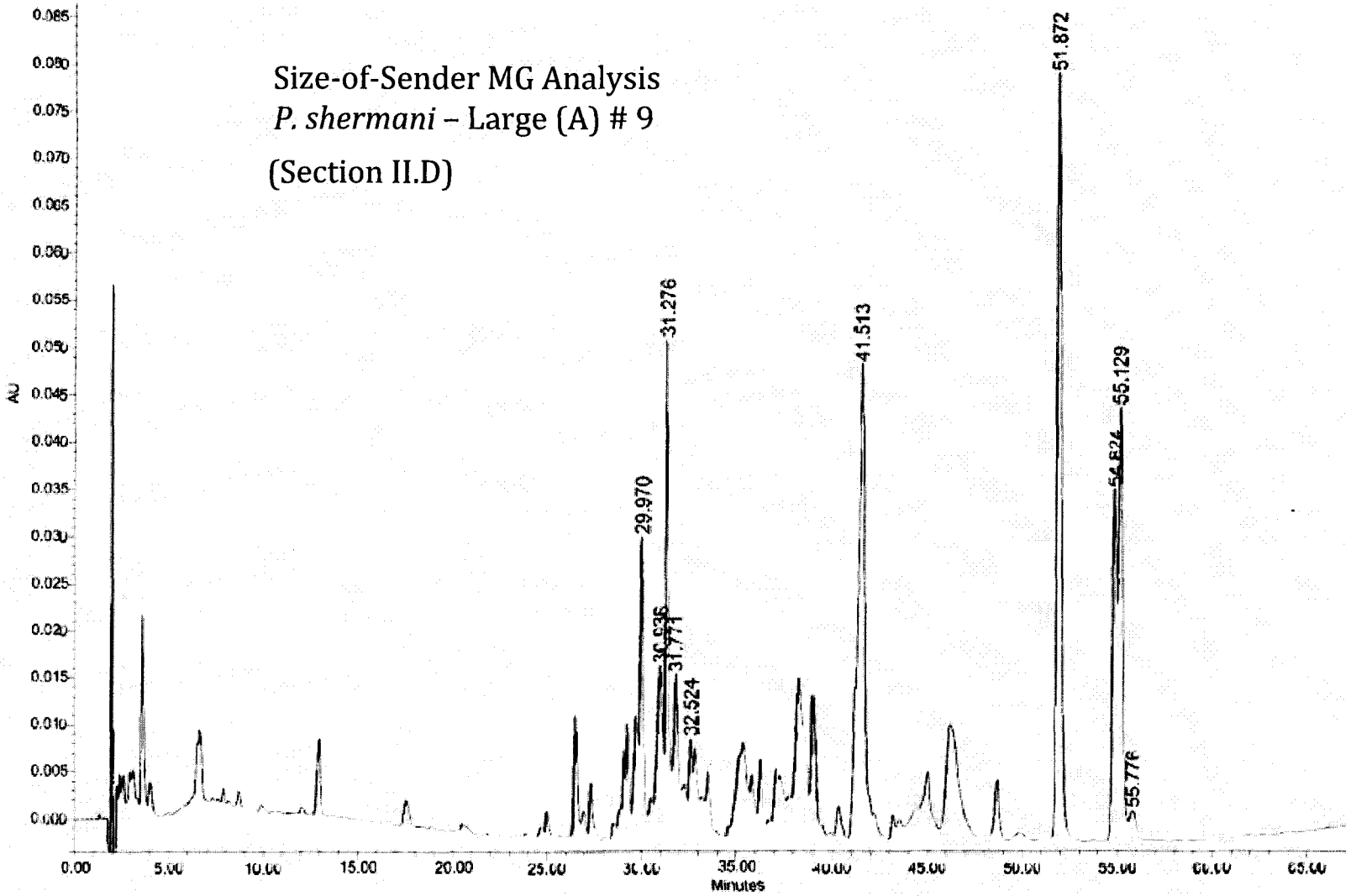
Size-of-Sender MG Analysis
P. shermani – Large # (A) 6
(Section II.D)



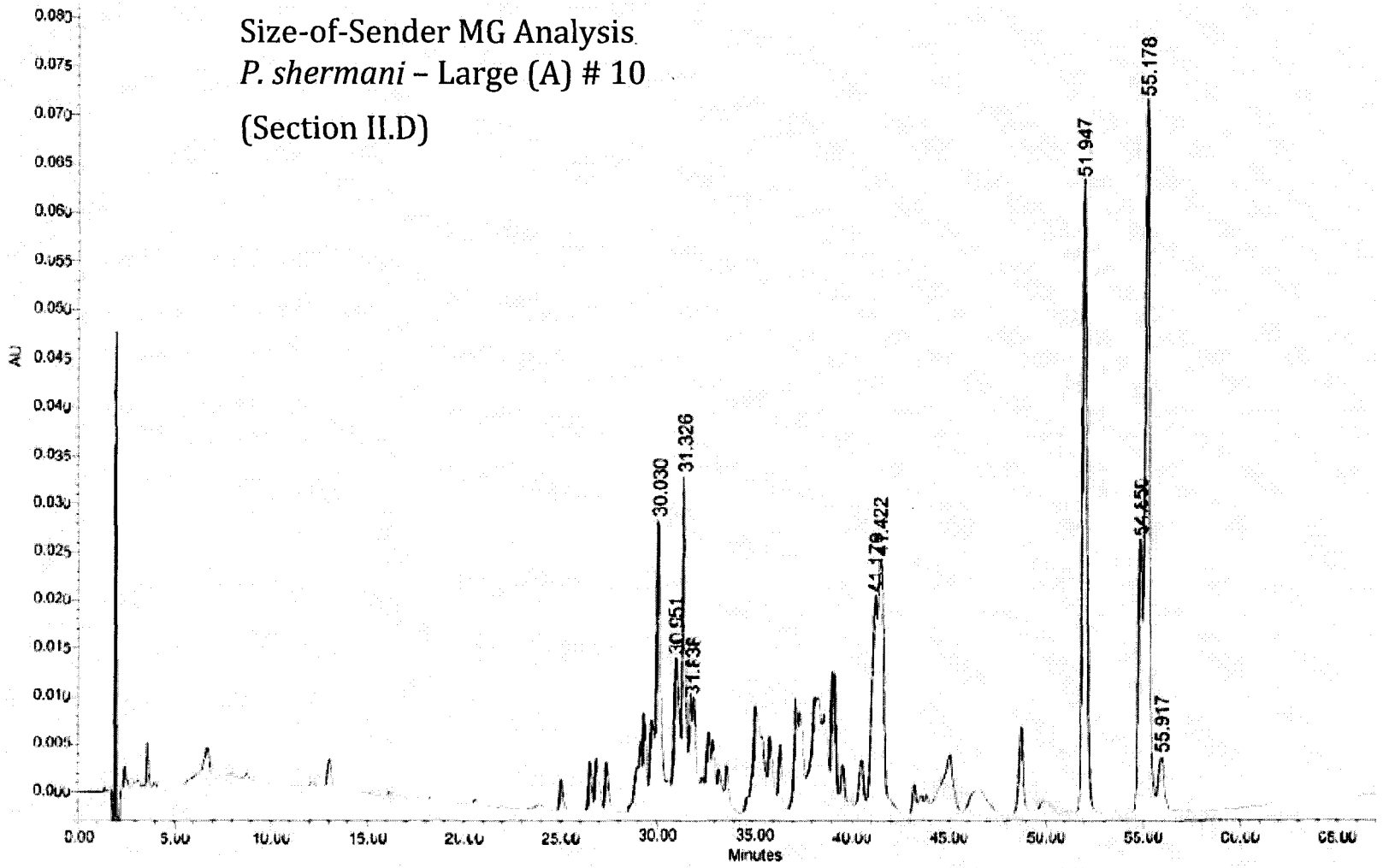

Size-of-Sender MG Analysis
P. shermani – Large # (A) 7
(Section II.D)
AU
Minutes
30.743
29.957
31.255
41.452
51.991
55.124
55.861

Size-of-Sender MG Analysis
P. shermani – Large (A) # 8
(Section II.D)




Size-of-Sender MG Analysis
P. shermani – Large (A) # 9
(Section II.D)
AU
Minutes
29.970
31.276
32.524
41.513
51.872
55.129

Size-of-Sender MG Analysis
P. shermani – Large (A) # 10
(Section II.D)

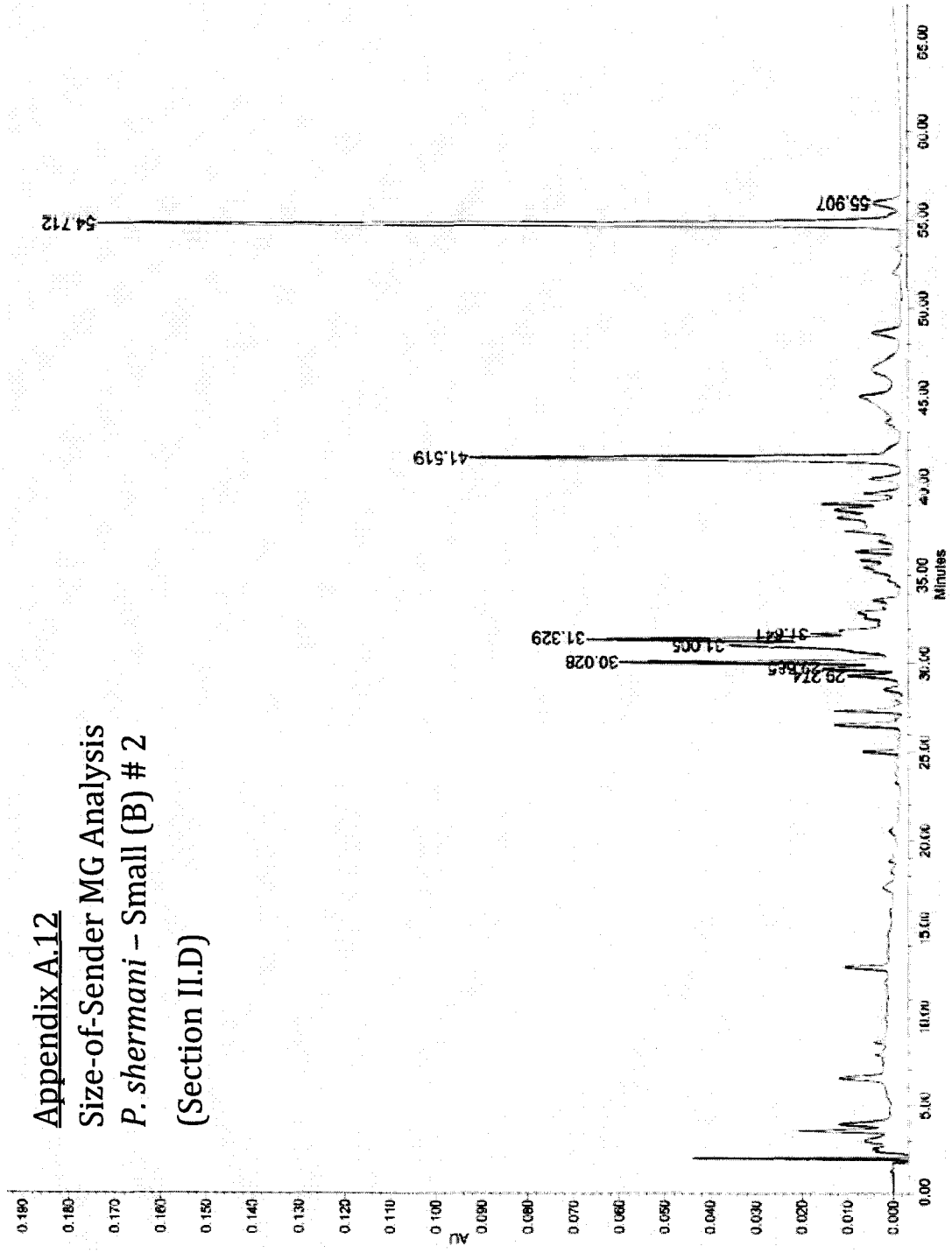


Appendix A.12

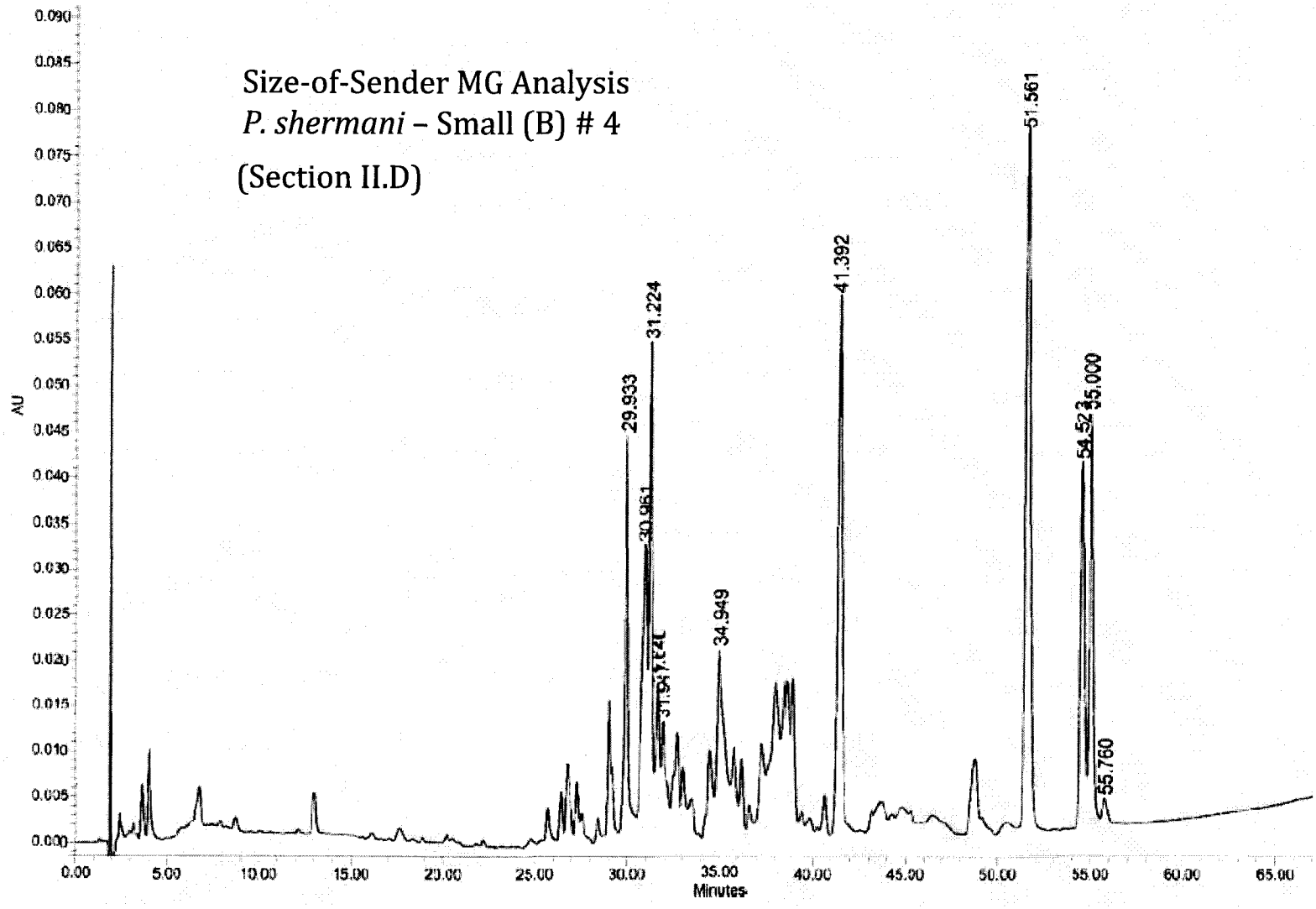
Size-of-Sender MG Analysis

P. shermani – Small (B) # 2

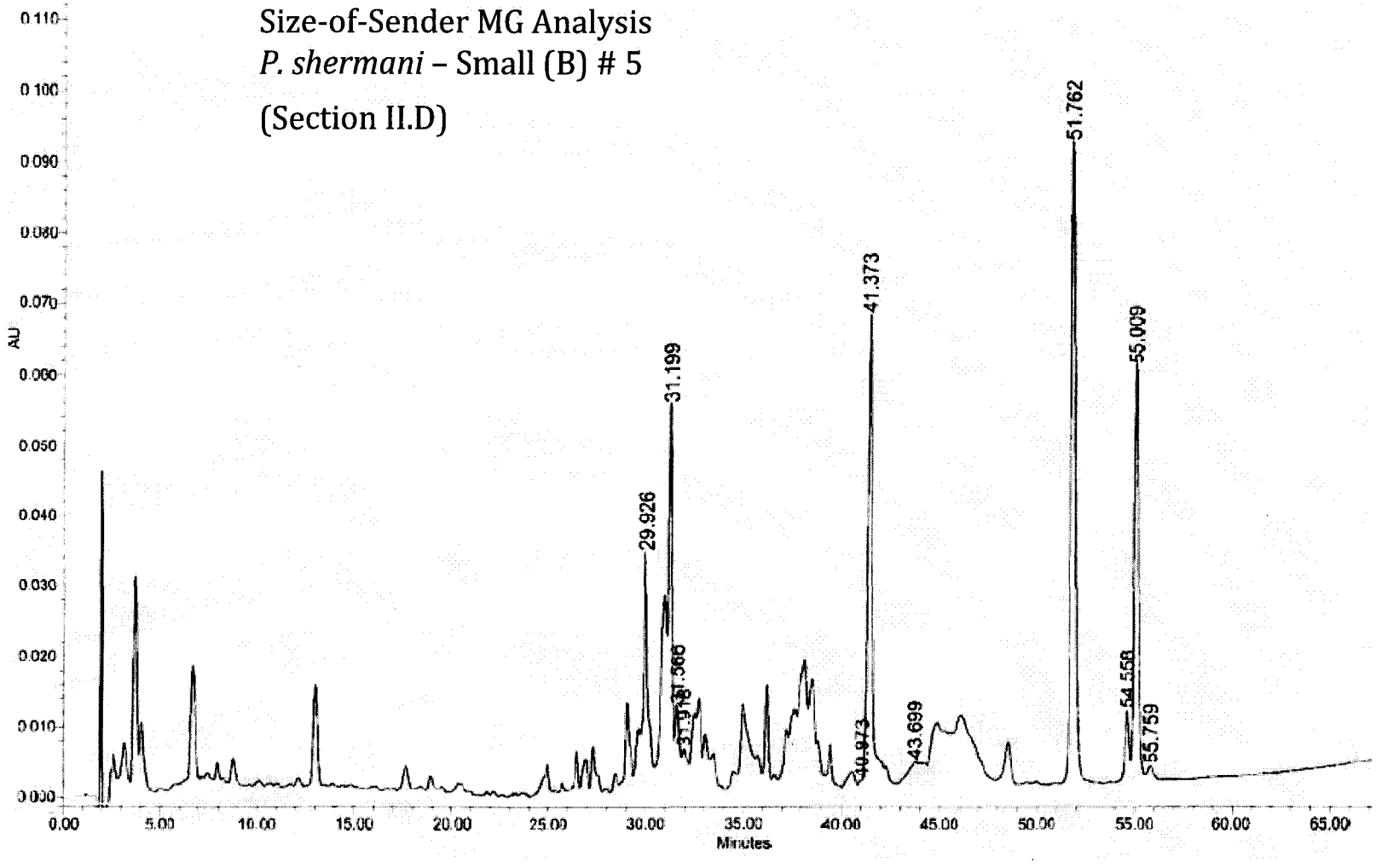
(Section II.D)



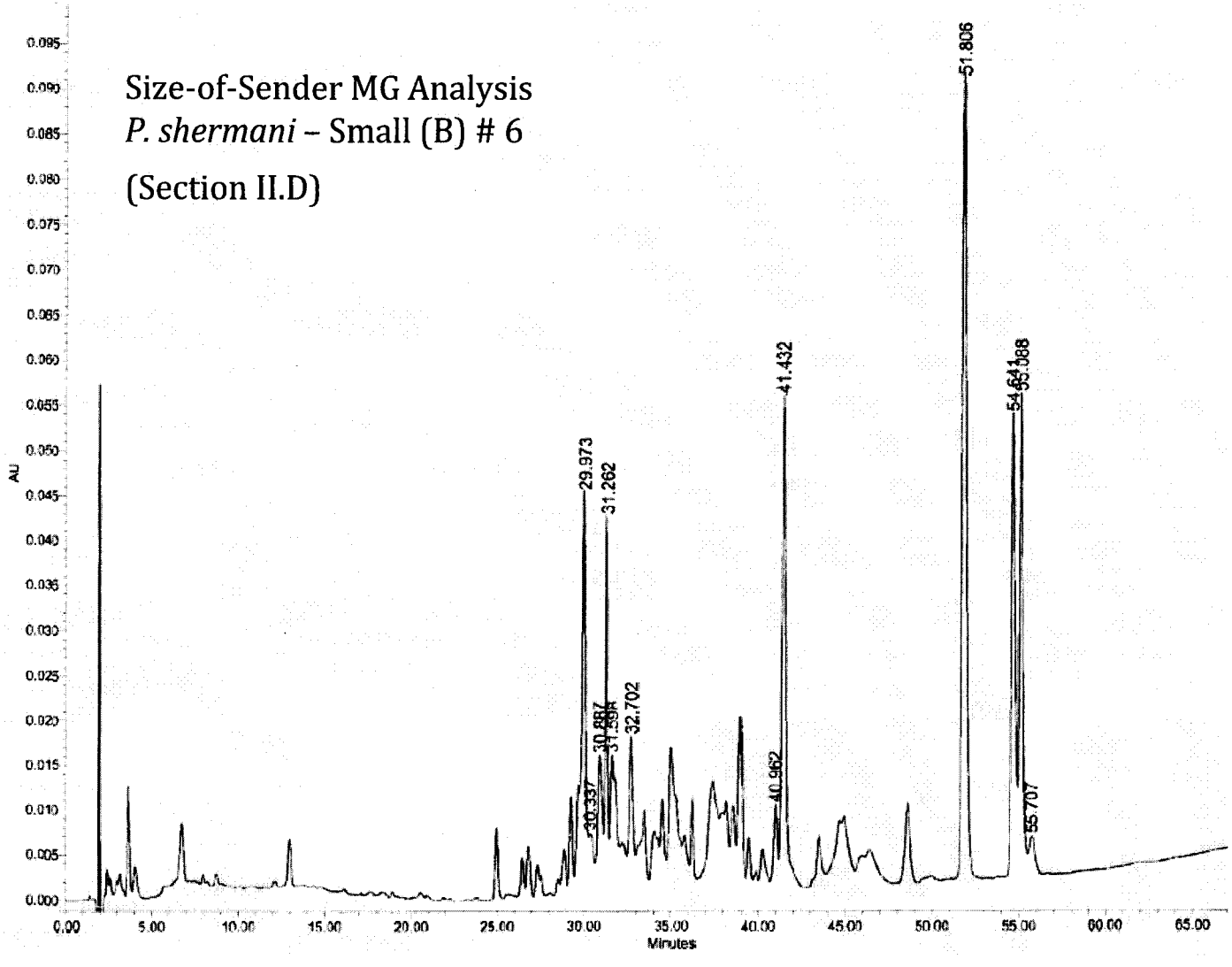
Size-of-Sender MG Analysis
P. shermani – Small (B) # 4
(Section II.D)



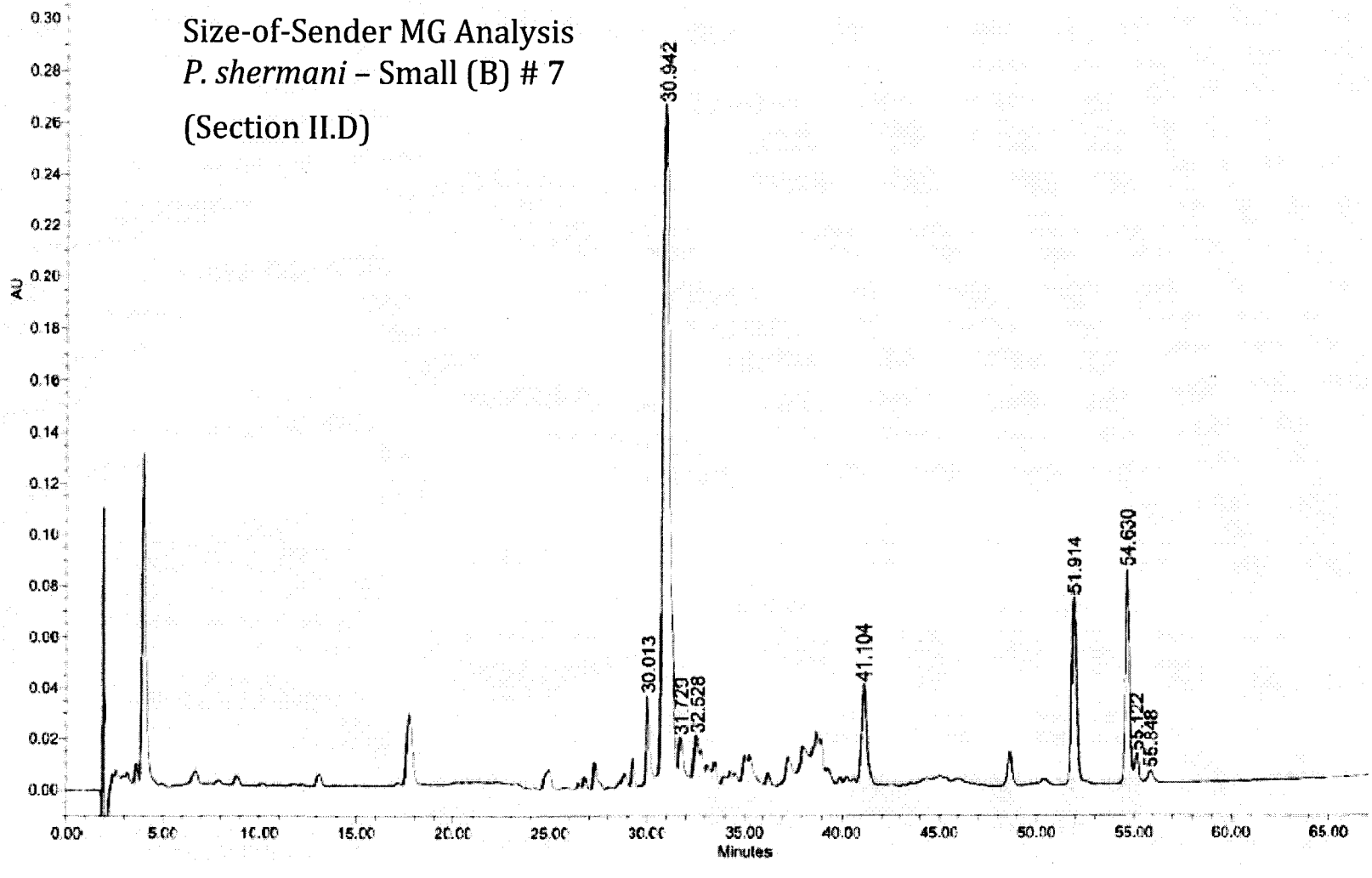
Size-of-Sender MG Analysis
P. shermani – Small (B) # 5
(Section II.D)

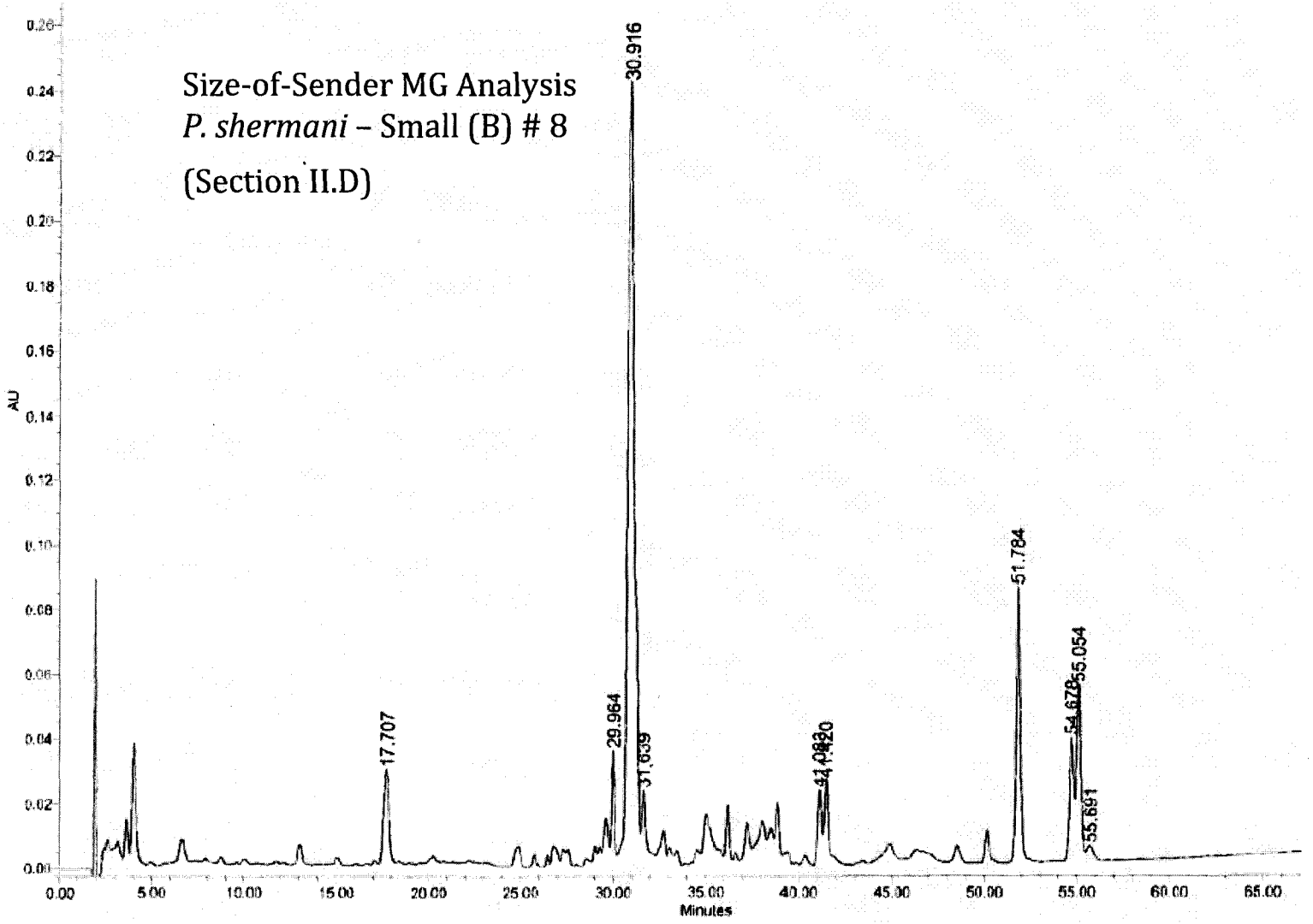


Size-of-Sender MG Analysis
P. shermani – Small (B) # 6
(Section II.D)



Size-of-Sender MG Analysis
P. shermani – Small (B) # 7
(Section II.D)

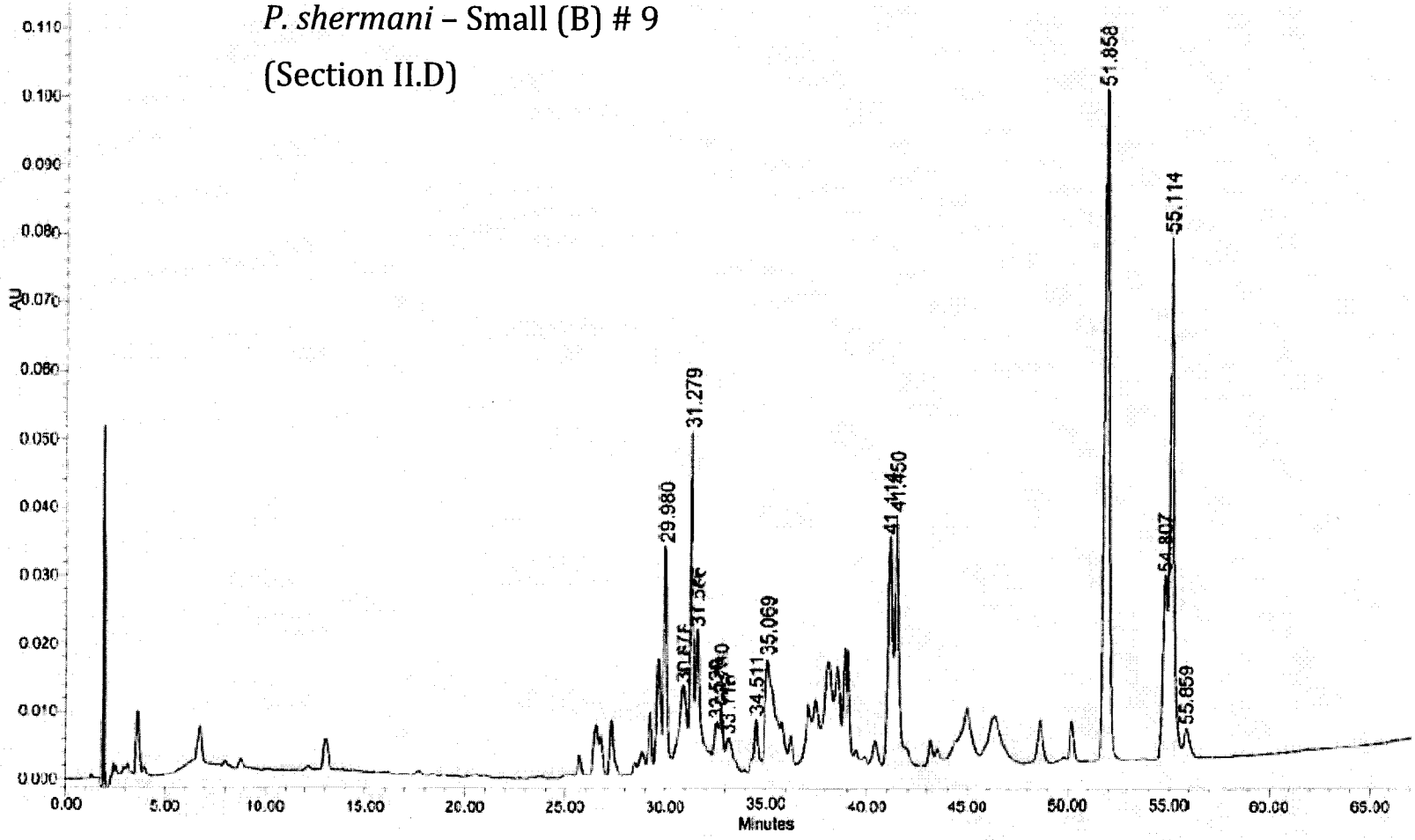



Size-of-Sender MG Analysis
P. shermani – Small (B) # 8
(Section II.D)
AU
Minutes
17.707
29.964
30.916
31.639
41.080
41.820
51.784
54.678
55.054
55.691
0.00
5.00
10.00
15.00
20.00
25.00
30.00
35.00
40.00
45.00
50.00
55.00
60.00
65.00

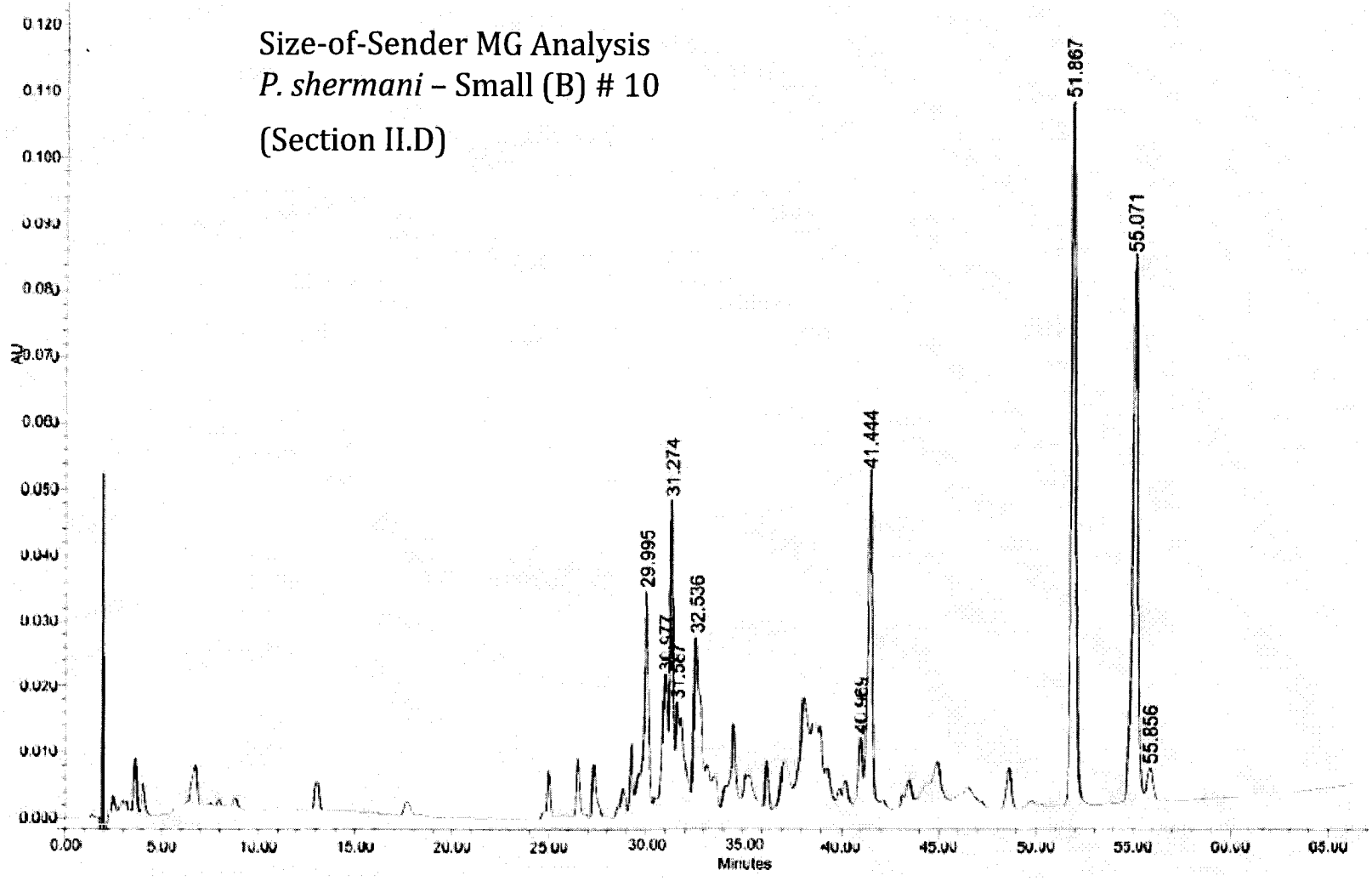
Size-of-Sender MG Analysis

P. shermani – Small (B) # 9

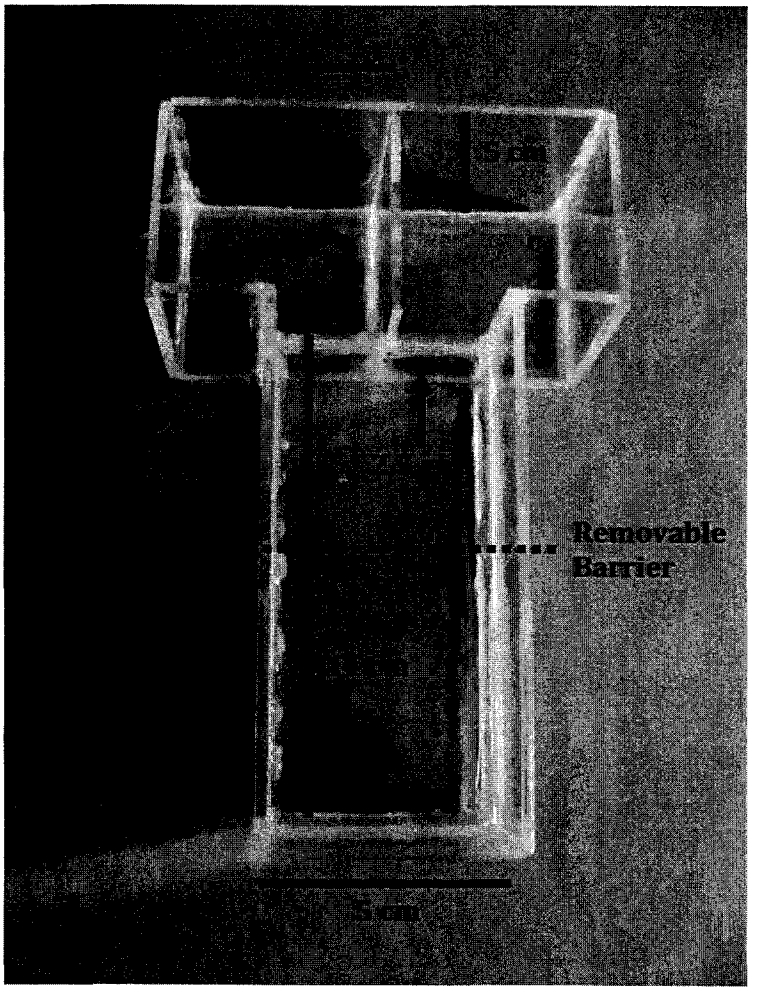
(Section II.D)



Size-of-Sender MG Analysis
P. shermani – Small (B) # 10
(Section II.D)

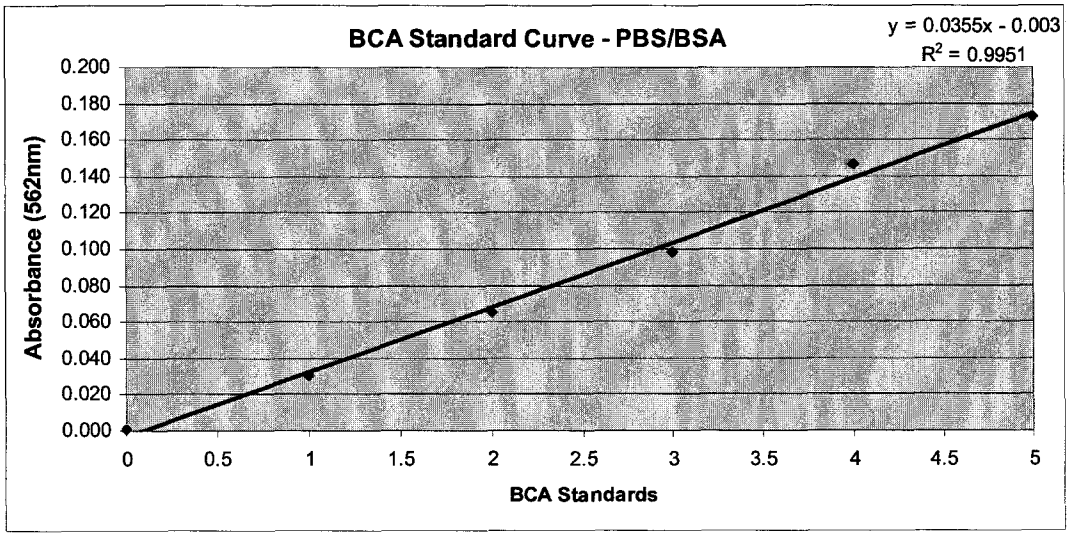


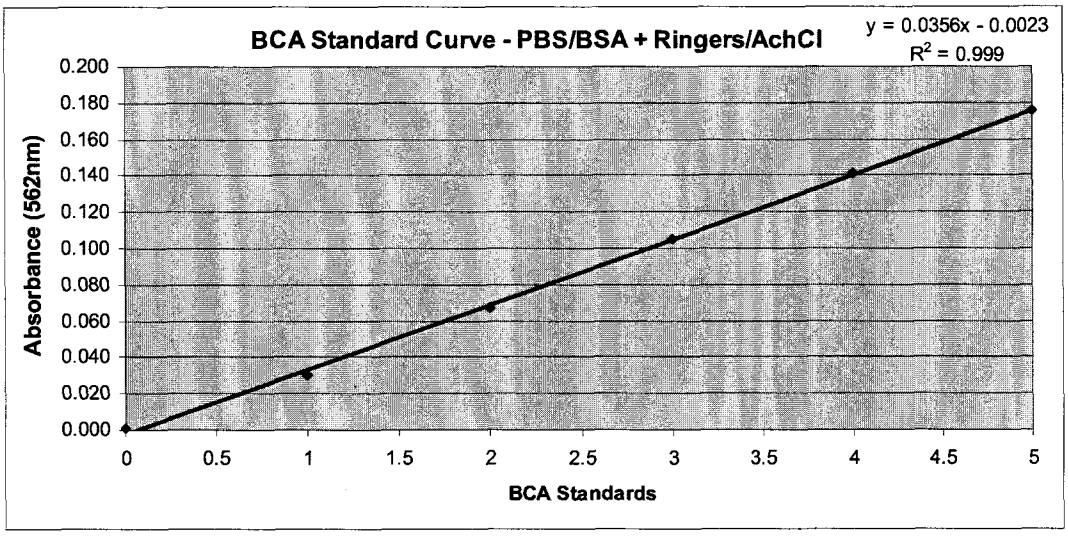
APPENDIX B: DIAGRAM OF EXPERIMENTAL Y-MAZE



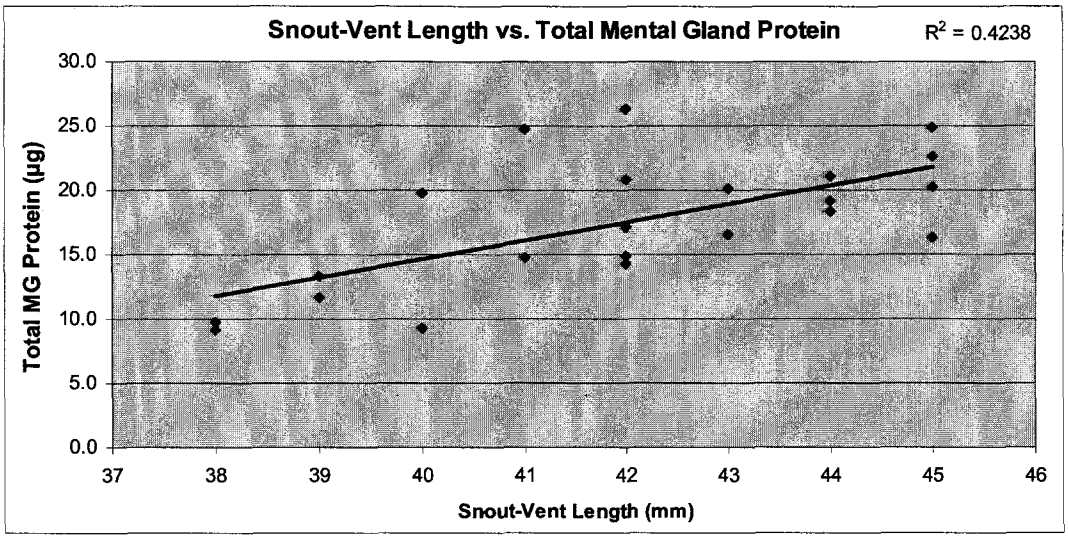
Appendix B. The experimental Y-maze used in the behavioral analysis of Section II.A.2-3. Dimensions for all sides are shown as solid lines, with the exception of the drop-off into the experimental chambers (see arrow). The cover of the Y-maze (not depicted) contained a small slit through which a removable barrier could be raised and lowered. This was used for containing the focal female in the initial region of the maze for the habituation period, after which the barrier was lifted and the trial began.

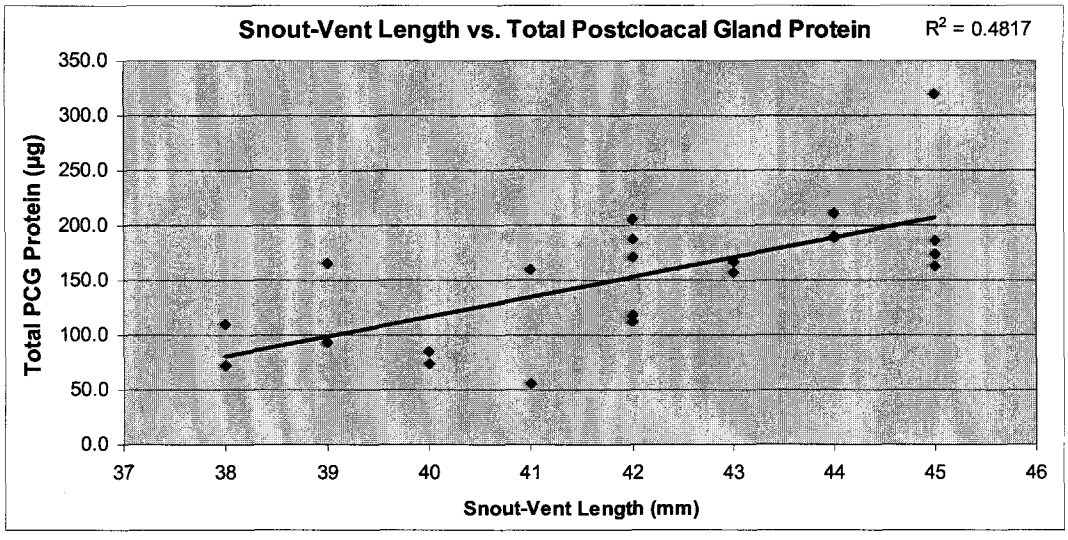
APPENDIX C: BCA Standard Curve Control



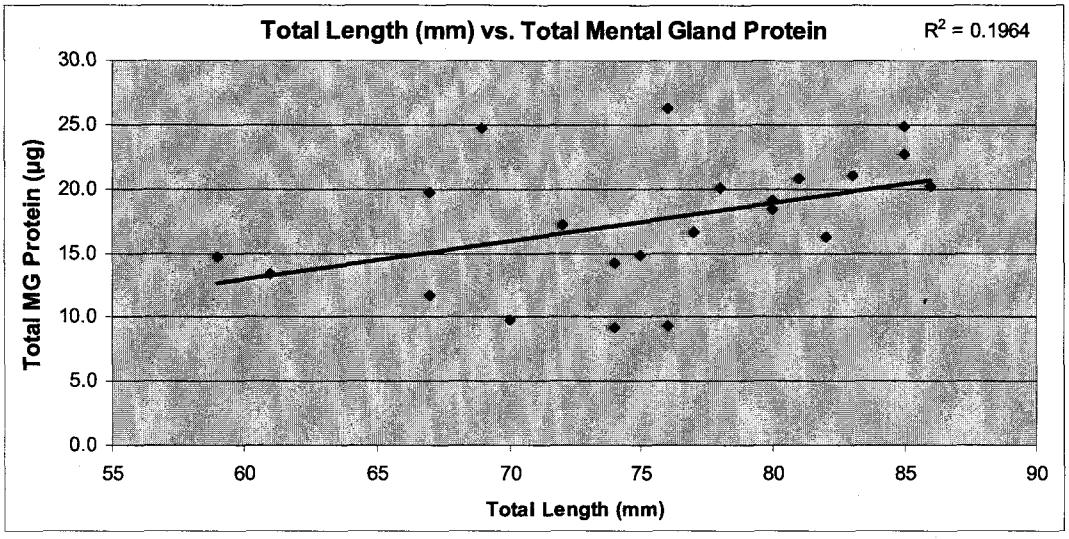


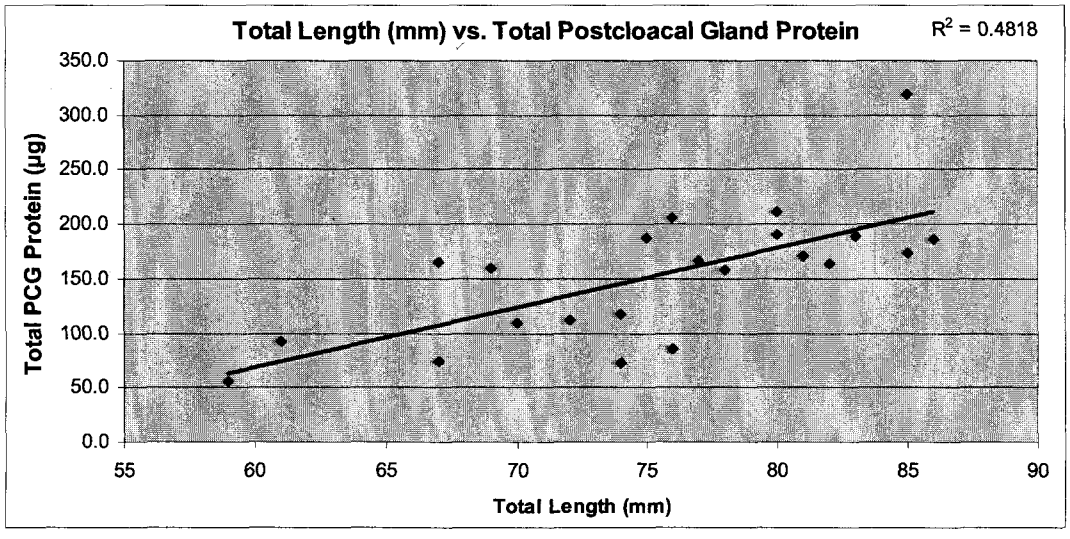
APPENDIX D.1: Additional Correlations (SVL vs. Gland Protein)



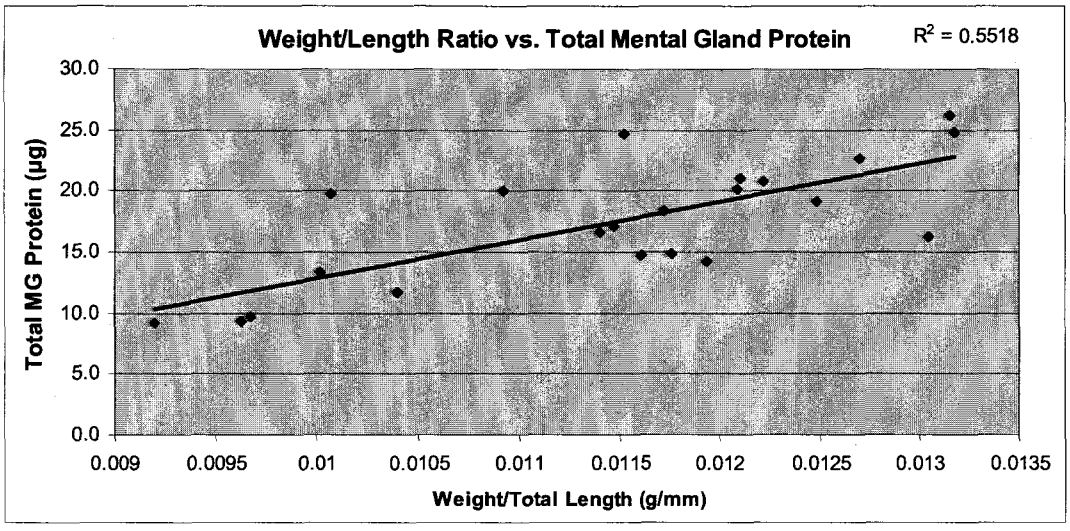


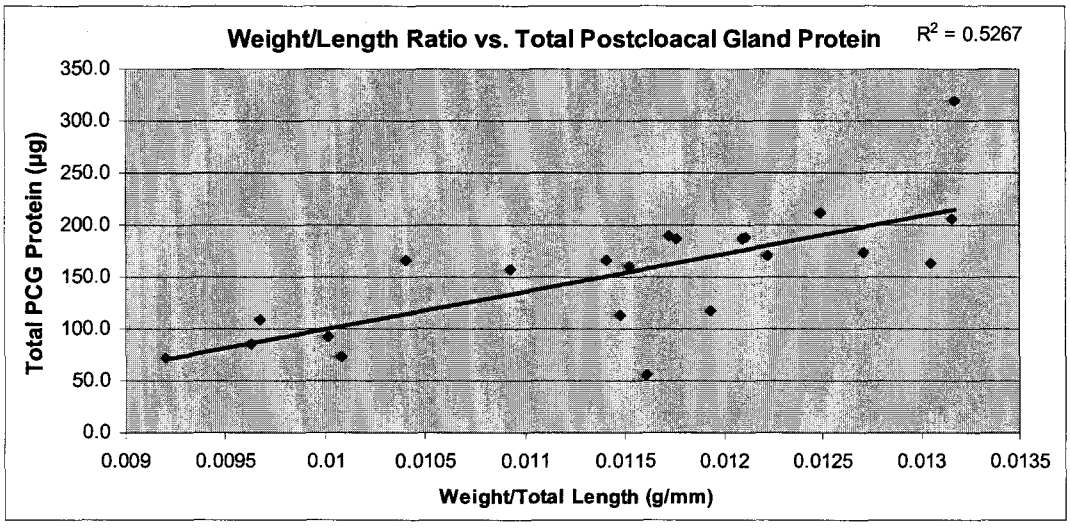
APPENDIX D.2: Additional Correlations (TL vs. Gland Protein)



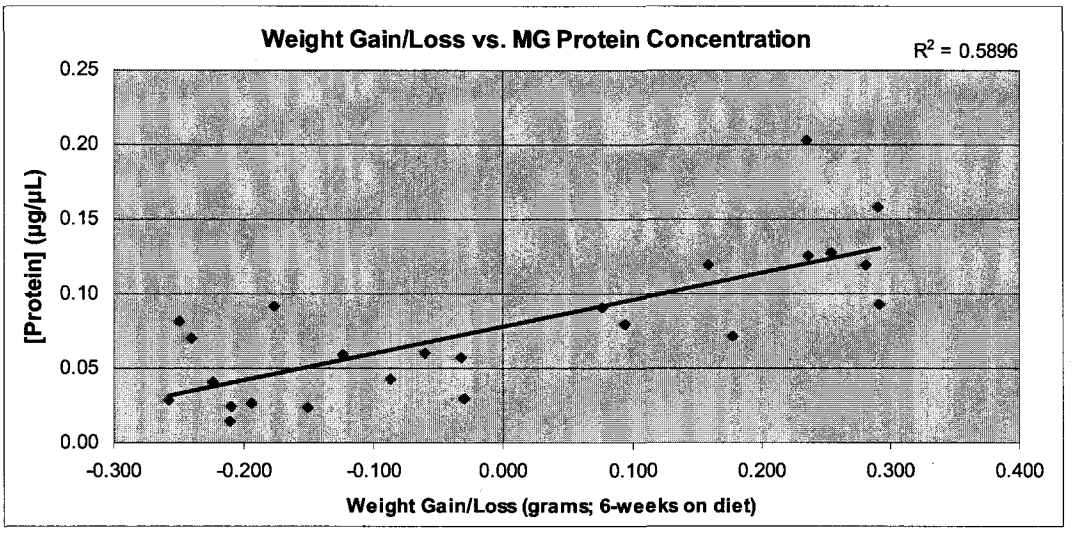


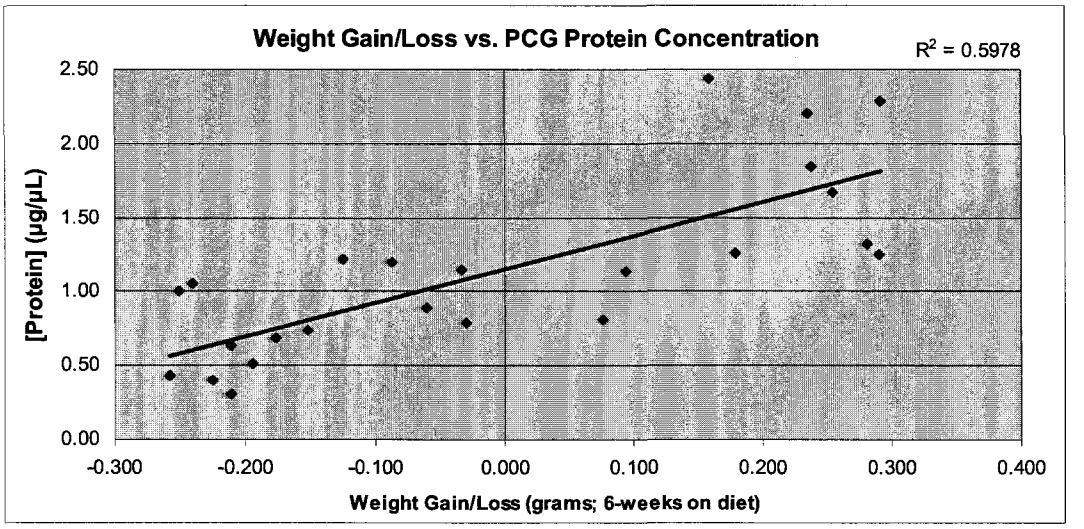
APPENDIX D.3: Additional Correlations (Weight/Length Ratio vs. Gland Protein)



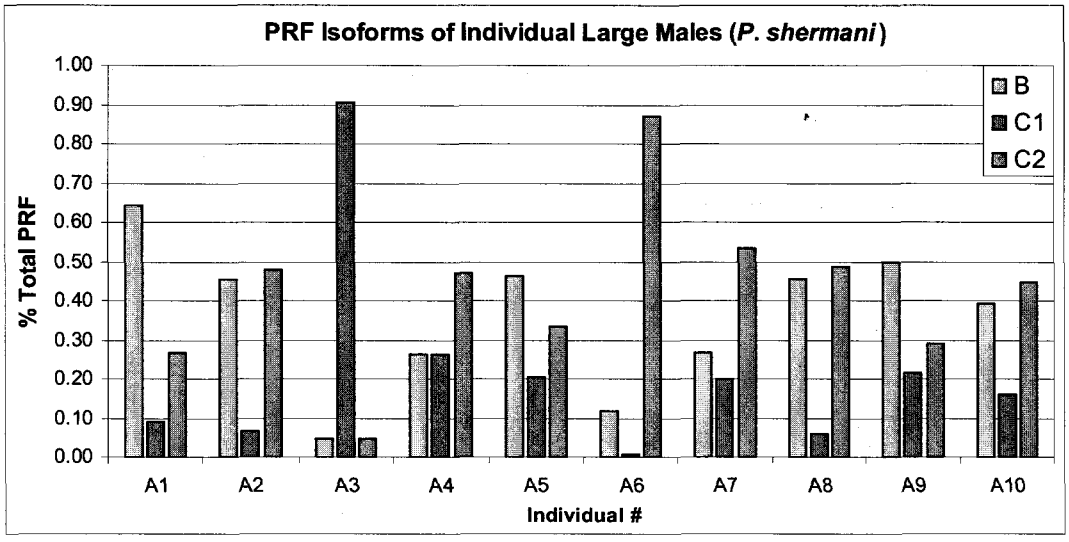


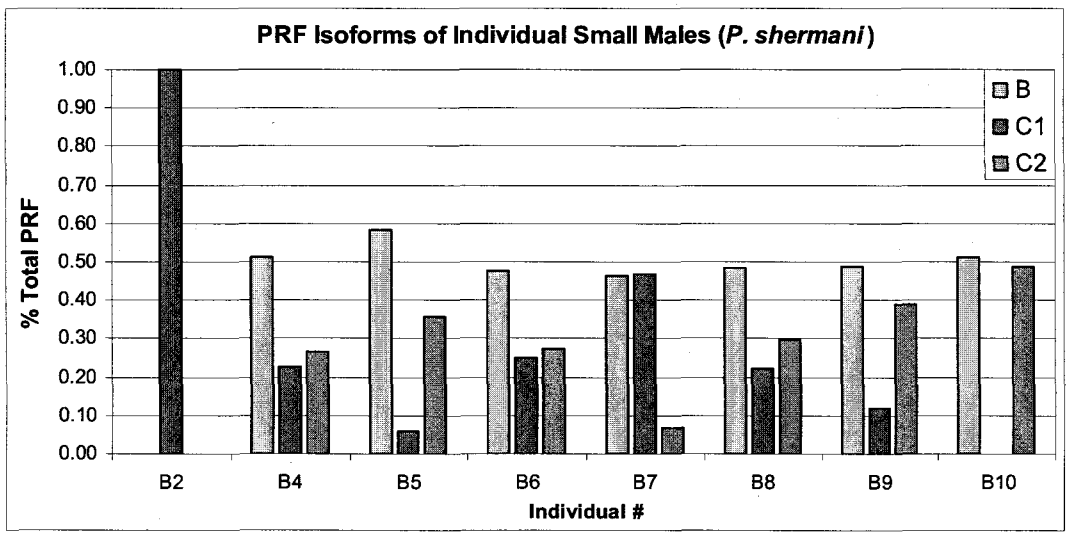
APPENDIX D.4: Additional Correlations (Weight Gain/Loss vs. Gland Protein)



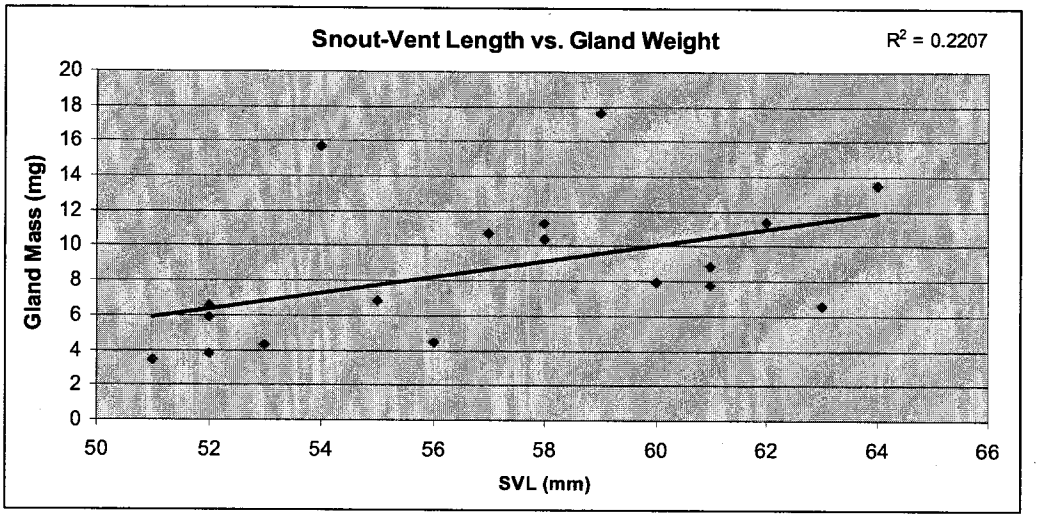


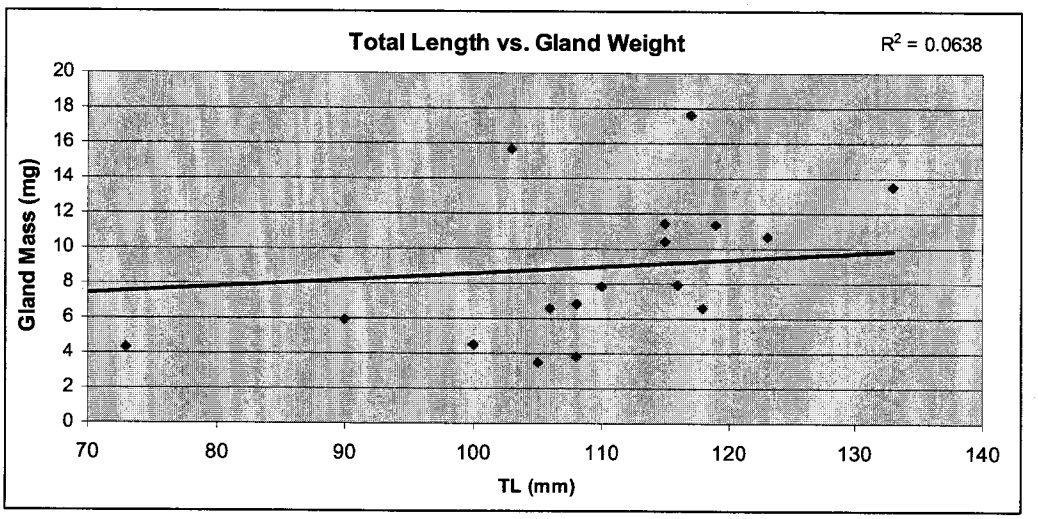
APPENDIX E: Individual *P. shermani* PRF Isoform Ratios

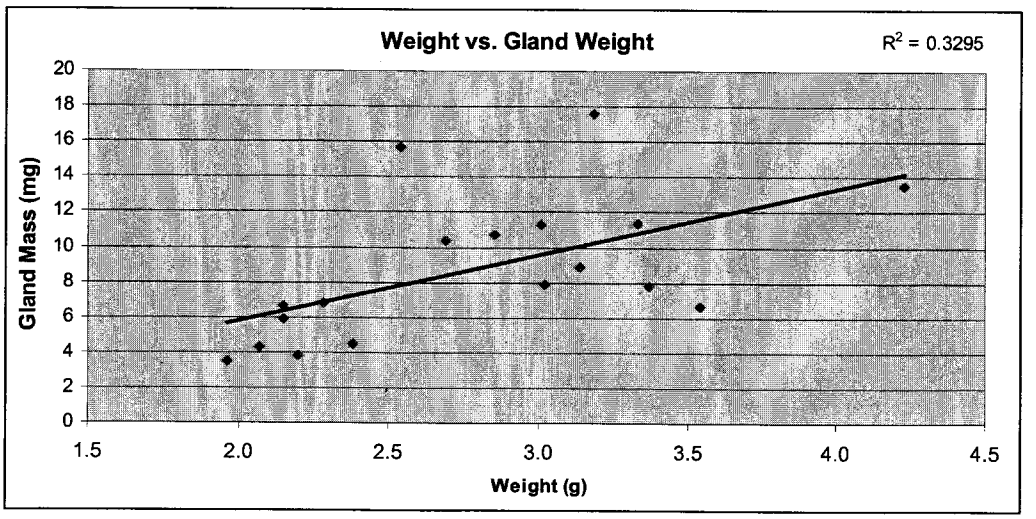




APPENDIX F: Body Size/Gland Size Relationships (*P. shermani*)







APPENDIX G: IACUC Approval Forms

University *of* New Hampshire

Research Conduct and Compliance Services, Office of Sponsored Research
Service Building, 51 College Road, Durham, NH 03824-3585
Fax: 603-862-3564

30-Oct-2007

Scott, Michelle
Zoology, Rudman Hall
Durham, NH 03824

IACUC #: 071005
Project: Pheromone Analysis of Male Salamanders with Differing Diets
Category: B
Approval Date: 19-Oct-2007

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol submitted for this study under Category B on Page 5 of the Application for Review of Vertebrate Animal Use in Research or Instruction - *the study involves either no pain or potentially involves momentary, slight pain, discomfort or stress.*

Approval is granted for a period of three years from the approval date above. Continued approval throughout the three year period is contingent upon completion of annual reports on the use of animals. At the end of the three year approval period you may submit a new application and request for extension to continue this project. Requests for extension must be filed prior to the expiration of the original approval.

Please Note:
1. All cage, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. A Medical History Questionnaire accompanies this approval; please copy and distribute to all listed project staff who have not completed this form already. Completed questionnaires should be sent to Dr. Gladi Porsche, UNH Health Services.

If you have any questions, please contact either Roger Wells at 862-2726 or Julie Simpson at 862-2003.

For the IACUC,

Jessica A. Bolker, Ph.D.
Chair

University *of* New Hampshire

Research Conduct and Compliance Services, Office of Sponsored Research
Service Building, 51 College Road, Durham, NH 03824-3585
Fax: 603-862-3564

04-Jun-2008

Scott, Michelle
Zoology, Rudman Hall
Durham, NH 03824

IACUC #: 080502
Project: Pheromone Analyses of Male Red-backed Salamanders
Category: C
Approval Date: 16-May-2008

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol submitted for this study under Category C on Page 5 of the Application for Review of Vertebrate Animal Use in Research or Instruction - *the research potentially involves minor short-term pain, discomfort or distress which will be treated with appropriate anesthetics/analgesics or other assessments.* The IACUC made the following comment(s) on this protocol:

1. If the investigator only plans to have her collaborator identify the proteins in these glands, then 20 animals is adequate. If, however, she thinks that her collaborator will be able to determine the primary amino acid structure, then 20 animals is not enough. This was unclear in the protocol.
2. The 20 animals for the pheromone definition study should not be returned to source since the glands have been removed.

Approval is granted for a period of three years from the approval date above. Continued approval throughout the three year period is contingent upon completion of annual reports on the use of animals. At the end of the three year approval period you may submit a new application and request for extension to continue this project. Requests for extension must be filed prior to the expiration of the original approval.

Please Note:
1. All cage, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. A Medical History Questionnaire accompanies this approval; please copy and distribute to all listed project staff who have not completed this form already. Completed questionnaires should be sent to Dr. Gladi Porsche, UNH Health Services.

If you have any questions, please contact either Dean Elder at 862-4629 or Julie Simpson at 862-2003.

For the IACUC,

Jessica A. Bolker, Ph.D.
Chair

cc: File
Chouinard, Adam